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COMPOSITION FOR INSERTING NUCLEIC ACID COMPLEXES INTO HIGHER EUCARYOTIC CELLS

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(57) Claim

1. A composition for the transfection of higher eucaryotic cells with a complex of nucleic acid and a substance having affinity for nucleic acid, which substance is optionally coupled with an internalizing factor for said cells, characterized in that said composition contains an endosomolytic agent which has the ability of being internalized into the cells which are to be transfected, either per se or as a component of the nucleic acid complex, and of releasing the contents of the endosome, in which the complex is located after entering the cell, into the cytoplasm.

81. A transfection kit containing a carrier unit in which there are two or more containers, a first container containing a substance having an affinity for nucleic acid which is optionally coupled to an

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internalising factor for a higher eucaryotic cell and a second container contains a substance having an affinity for nucleic acid which is coupled to an endosomolytic agent capable of penetrating into higher eucaryotic cells as a component of a nucleic acid complex and releasing the contents of endosomes into the cytoplasm.

PATENTS ACT 1990
COMMONWEALTH OF AUSTRALIA

NOTICE OF ENTITLEMENT

I/We BOEHRINGER INGELHEIM INTERNATIONAL GmbH and THE UNIVERSITY OF NORTH CAROLINA, the Applicant/Nominated Person in respect of Application No. 26526/92 state the following:

The Nominated Persons are entitled to the grant of the patent because Boehringer Ingelheim International GmbH would on the grant of a patent to inventors E Wagner, M Cotten, K Zatloukal, C Plank, M Birnstiel, B Oberhauser and W Schmidt be entitled to have the patent assigned to it; The University of North Carolina derives title from inventor D Curiel by assignment.

The Nominated Persons are entitled to claim priority from the applications listed in the Declaration under Article 8 of the PCT because Boehringer Ingelheim International GmbH derives title by agreement from inventors E Wagner, M Cotten, K Zatloukal, C Plank, M Birnstiel, B Oberhauser and W Schmidt and The University of North Carolina is the Assignee of inventor D Curiel; Boehringer Ingelheim International GmbH and The University of North Carolina are the applicants in respect of the basic applications listed in the Declaration under Article 8 of the PCT and the applications were the first applications made in a convention country in respect of the invention.

DATED this 17th day of May, 1996.

a member of the firm of
DAVIES COLLISON CAVE
and on behalf of the applicant(s)

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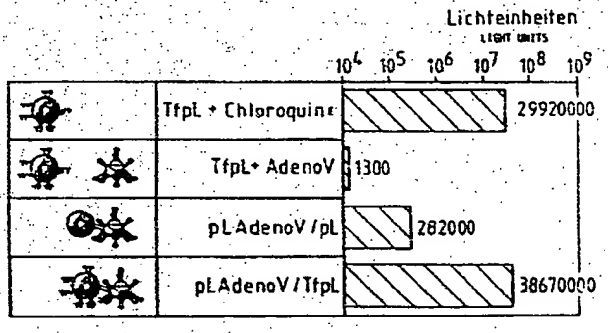
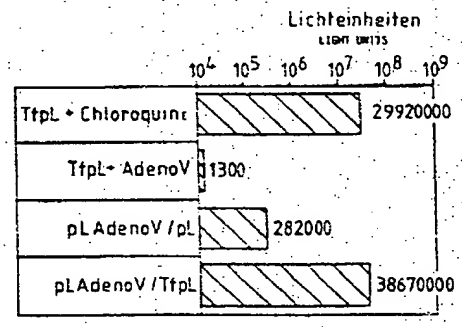
(54) Title: COMPOSITION FOR INSERTING NUCLEIC ACID COMPLEXES INTO HIGHER EUCARYOTIC CELLS

(54) Bezeichnung: ZUSAMMENSETZUNG FÜR DAS EINBRINGEN VON NUKLEINSÄURE-KOMPLEXEN IN HÖHERE EUKARYOTISCHE ZELLEN





(57) Abstract

A composition for transfecting higher eucaryotic cells with a complex of nucleic acid and a nucleic acid-affine substance is possibly conjugated with an internalization factor for such cells. The composition contains an endosomolytic agent that is a free or bound virus or virus component or a viral or non-viral peptide with endosomolytic properties.

(57) Zusammenfassung

Zusammensetzung für die Transfektion von höheren eukaryotischen Zellen mit einem Komplex aus Nukleinsäure und Nukleinsäure-affiner Substanz, die gegebenenfalls mit einem Internalisierungsfaktor für diese Zellen konjugiert ist. Die Zusammensetzung enthält ein endosomolytisches Mittel, das entweder ein freies oder ein gebundenes Virus oder eine Viruskomponente oder ein virales oder nicht-virales Peptid mit endosomolytischen Eigenschaften ist.

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Composition for Introducing Nucleic Acid Complexes Into Higher Eucaryotic Cells

The invention relates to the introduction of nucleic acids into higher eucaryotic cells.

There is a need for an efficient system for introducing nucleic acid into living cells particularly in gene therapy. Genes are transferred into cells in order to achieve in vivo synthesis of therapeutically effective genetic products, e.g. in order to replace the defective gene in the case of a genetic defect. "Conventional" gene therapy is based on the principle of achieving a lasting cure by a single treatment. However, there is also a need for methods of treatment in which the therapeutically effective DNA (or mRNA) is administered like a drug ("gene therapeutic agent") once or repeatedly as necessary. Examples of genetically caused diseases in which gene therapy represents a promising approach are hemophilia, beta-thalassaemia and "Severe Combined Immune Deficiency" (SCID), a syndrome caused by the genetically induced absence of the enzyme adenosine deaminase. Other possible applications are in immune regulation, in which humoral or intracellular immunity is achieved by the administration of functional nucleic acid which codes for a secreted protein antigen or for a non-secreted protein antigen, by means of a vaccination. Other examples of genetic defects in which a nucleic acid which codes for the defective gene can be administered, e.g. in a form individually tailored to the particular requirement, include muscular dystrophy (dystrophin gene), cystic fibrosis (cystic fibrosis transmembrane conductance regulator gene), hypercholesterolemia (LDL receptor gene). Gene therapy methods are also potentially of use when hormones,

growth factors or proteins with a cytotoxic or immune-modulating activity are to be synthesized in the body.

Gene therapy also appears promising for the treatment of cancer by administering so-called "cancer vaccines". In order to increase the immunogenicity of tumor cells, they are altered to render them either more antigenic or to make them produce certain immune modulating substances, e.g.cytokines, in order to trigger an immune response. This is accomplished by transfecting the cells with DNA coding for a cytokine, e.g. IL-2, IL-4, IFN-gamma, TNF-alpha. To date, most gene transfer into autologous tumor cells has been accomplished via retroviral vectors.

The technologies which are hitherto most advanced for the administration of nucleic acids in gene therapy, make use of retroviral systems for transferring genes into the cells (Wilson et al., 1990, Kasid et al., 199-0). However, the use of retroviruses is problematic because it brings, at least to a small degree, the danger of side effects such as infection with the virus (by recombination with endogenous viruses or contamination with helper viruses and possible subsequent mutation into the pathogenic form) or the formation of cancer. Moreover, the stable transformation of the somatic cells in the patient, as achieved with retroviruses, is not desirable in every case because it might make the treatment difficult to reverse, e.g. if side effects occur. Moreover, with this type of therapy, it is difficult to obtain a high enough titer to infect enough cells.

Nucleic acids as therapeutically effective substances are also used to inhibit specific cell functions, e.g. antisense RNAs and DNAs have proved effective in the selective inhibition of specific gene sequences. Their mode of activity enables them to be used as therapeutic agents for blocking the expression of certain genes (such as deregulated oncogenes or viral

genes) *in vivo*. It has already been shown that short antisense oligonucleotides can be imported into cells and exert their inhibiting effect therein (Zamecnik *et al.*, 1986), even if their intracellular concentration is low, caused, *inter alia*, by their restricted uptake by the cell membrane as a result of the strong negative charge of the nucleic acids.

Another approach to the selective inhibition of genes is the use of ribozymes. Again there is the need to ensure the highest possible concentration of active ribozymes in the cell, transportation into the cell being one of the limiting factors.

Application of gene therapy for achieving intracellular immunity involves transduction of genes which protect against viruses, so-called "protective genes", e.g. transdominant mutants of genes coding for viral proteins, or DNA molecules coding for so-called RNA decoys.

There is consequently a need for methods of enabling the expression of DNA in the cell.

Numerous solutions have been proposed for improving the transfer of nucleic acids into living cells, which is one of the limiting factors affecting their therapeutic use.

Various techniques are known for gene transformation of mammalian cells *in vitro*, but their use *in vivo* is limited (these include the introduction of DNA by means of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran or the calcium phosphate precipitation method).

In recent times, recombinant viral vectors have been developed to bring about the transfer of genes by using the efficient entry mechanisms of their parent viruses. This strategy was used in the construction of recombinant retroviral and adenoviral vectors in order to achieve a highly efficient gene transfer *in vitro* and *in vivo* (Berkner, 1988). Despite their efficiency,

these vectors are subject to restrictions in terms of the size and construction of the DNA which is transferred. Furthermore, these agents represent safety risks in view of the co-transfer of the viable viral gene elements of the original virus.

In order to circumvent these restrictions, alternative strategies for gene transfer have been developed, based on mechanisms which the cell uses for the transfer of macromolecules. One example of this is the transfer of genes into the cell via the extremely efficient route of receptor-mediated endocytosis (Wu and Wu, 1987, Wagner et al., 1990 and EP-A1 0388 758). This approach uses bifunctional molecular conjugates which have a DNA binding domain and a domain with specificity for a cell surface receptor (Wu and Wu, 1987, Wagner et al., 1990). If the recognition domain is recognized by the cell surface receptor, the conjugate is internalized by the route of receptor-mediated endocytosis, in which the DNA bound to the conjugate is also transferred. Using this method, it was possible to achieve gene transfer rates at least as good as those achieved with the conventional methods (Zenke et al., 1990).

It has been shown that using this system DNA transferred into the cell is expressed and, if a nucleic acid with an inhibitory effect is used, the inhibitory effect is not impaired by the transport system.

The PCT Application WO 91/17773 relates to a system for transporting nucleic acids with a specific activity for T-cells. This system makes use of cell surface proteins of the T-cell lineage, e.g. CD4, the receptor used by the HIV virus. The nucleic acid to be imported is complexed with a protein-polycation conjugate, the protein component of which is a protein capable of binding to the T-cell surface protein, e.g. CD4, and cells which express this surface protein are brought into contact with the resulting protein-

polycation/nucleic acid complexes. It has been shown that DNA transported into the cell by means of this system is expressed in the cell.

One feature common to both inventions is that they use specific cell functions to enable or facilitate the transfer of nucleic acid into the cell. In both cases, the uptake mechanisms take place with the participation of factors which are termed "internalizing factors" within the scope of the present invention. This term denotes factors which, being cell-type-specific in the narrower or wider sense, bind to the cell surface and are internalized, possibly with the cooperation of other factors (e.g. cell surface proteins). (In the case of the two inventions mentioned above, the internalizing factor is transferrin or a protein which binds to a T-cell surface antigen, e.g. an anti-CD4 antibody). The internalizing factor is conjugated with a substance of a polycationic nature which, by virtue of its affinity for nucleic acids, forms an association between the internalizing factor and the nucleic acid. (Substances of this kind are hereinafter referred to as "substances with an affinity for nucleic acid" or with regard to DNA, "DNA binding domain". If a substance of this kind as part of the conjugate forms a bond between the nucleic acid and an internalizing factor it is hereinafter referred to as a "binding factor").

In the course of these two inventions the optimum uptake of nucleic acid into the cell was achieved when the ratio of conjugate to nucleic acid was such that the internalizing factor-polycation/nucleic acid complexes were approximately electroneutral. Starting from this observation, the methods which use internalizing factor-binding factor/nucleic acid complexes to introduce nucleic acids into higher eucaryotic cells was improved.

A method for improving the efficiency of systems in which the uptake of nucleic acids is carried out by means of internalizing factors, was described by Wagner

et al., 1991a. In this method, the quantity of nucleic acid taken up into the cell is not reduced if part of the transferrin-polycation conjugate is replaced by non-covalently bound polycation; in certain cases, this may even increase the DNA uptake considerably.
Investigations into the molecular state of transferrin-polycation-plasmid DNA complexes produced with optimum ratios of DNA/conjugate showed that the plasmid DNA in the presence of the conjugates is condensed into toroidal structures (resembling doughnuts) with a diameter of about 80 to 100 nm).

Experiments conducted with proteins binding to T-cells as internalizing factor produced similar results.

The addition of free substances with an affinity for nucleic acid also results in an increase in the efficiency of the introduction system even when another substance with an affinity for nucleic acid is used as binding factor.

The complexes described by Wagner et al., 1991a, which are taken up into higher eucaryotic cells via endocytosis by means of internalizing factor, contain nucleic acid complexed with a conjugate of internalizing factor and binding factor. In addition, the complexes contain one or more substances with an affinity for nucleic acid which may possibly be identical to the binding factor, in a non-covalently bonded form, such that the internalization and/or expression of the nucleic acid achieved by means of the conjugate is increased, which would appear to be due primarily to a condensing effect but might possibly be due to other mechanisms.

Even if the rates of expression of the imported nucleic acid could be increased by this method, it is still subject to restrictions. The practicality of this system in a given context is not solely determined by the presence of the cell surface receptor relevant to the system; the limitations associated with the use of

this system are presumably a result of the fact that the conjugate-DNA complexes internalized in endosomes enter the lysosomes, where they are enzymatically degraded. In order to increase the proportion of nucleic acid which reaches the cell nucleus and is expressed there, as intended, attempts were made, in experiments preceding this invention, to carry out the transfection of the cells in the presence of substances which inhibit the enzymatic activity in the lysosomes, so-called lysosomatropic substances. By using this strategy, augmented expression of transferred DNA was achieved; however, the reactions achieved were highly variable, depending on the substance used; selected lysosomatropic substances brought about an increase in gene transfer, whereas others actually inhibited it. Thus, for example, it was found that the effective transfer of DNA depends on the presence of the weak base chloroquine (Zenke et al., 1990, Cotten et al., 1990). This effect achieved by means of chloroquine may not, or not exclusively, be due to the fact that chloroquine increases the pH in the lysosomes; it was found, from a number of different experiments, that other substances which, like chloroquine, have the ability to modulate pH, such as monensin, ammonium chloride or methylamine, could not replace chloroquine and in some experiments some of these substances even showed an inhibiting effect. It was further found that various target cells show different responses to the same substance having a lysosomatropic activity.

Since gene transfer by the physiological route, as represented by receptor-mediated endocytosis using nucleic acid complexes, has major advantages (non-toxic mechanism of passage through the cell membrane; the possibility of administering biologically active nucleic acids, such as genes, gene fragments or nucleic acids which specifically inhibit cellular functions, on a repeated or continuous basis; the possibility of cell-

specific targeting; the possibility of producing the conjugates in large quantities), there is a need to make this system more efficient.

The aim of the present invention was to improve the transfer of nucleic acid into higher eucaryotic cells. (The word "transfer" within the scope of this invention means, apart from the introduction of the nucleic acid complexes into the cell through the cell membrane, the transport of the complexes or the nucleic acid released therefrom within the cell until it reaches an appropriate site to be expressed). The higher eucaryotic cells are well known and do not include yeast. (Watson et al., (1987)).

Many types of virus [illegible] effect their entry into the eucaryotic host by means of mechanisms which correspond in principle to the mechanism of receptor-mediated endocytosis. Virus infection based on this mechanism generally begins with the binding of virus particles to receptors on the cell membrane. After this, the virus is internalized into the cell. This internalizing process follows a certain route, corresponding to the entrance of physiological ligands or macromolecules into the cell: first of all, the receptors on the cell surface arrange themselves in groups, to form a so-called "coated pit", and the membrane is inverted inwardly and forms a vesicle surrounded by a coating. After this vesicle has rid itself of its clathrin coat, acidification takes place inside it by means of a proton pump located in the membrane. This triggers the release of the virus from the endosome. Depending on whether the virus has a lipid coat or not, two types of virus release from the endosome were taken into account: in the case of so-called "naked" viruses (e.g. adenovirus, poliovirus, rhinovirus) it was suggested that the low pH causes changes in conformation in virus proteins. This exposes hydrophobic domains which are not accessible at the physiological pH. These domains thus acquire the

ability to interact with the endosome membrane and thereby cause the release of the virus genome from the endosome into the cytoplasm. As for viruses with a lipid coat (e.g. vesicular stomatitis virus, Semliki Forest virus, influenza virus) it is presumed that the low pH modifies the structure or conformation of some virus proteins, thereby promoting the fusion of the virus membrane with the endosome membrane. Viruses which penetrate into the cell by means of this mechanism have certain molecular peculiarities which enable them to break up the endosome membrane in order to gain entry into the cytoplasm.

Other viruses, e.g. the coated viruses Sendai, HIV and some strains of Moloney leukaemia virus, or the uncoated viruses SV40 and polyoma, do not need a low pH-milieu for penetration into the cell; they can either bring about fusion with the membrane directly on the surface of the cell (Sendai virus, possibly HIV) or they are capable of triggering mechanisms for breaking up the cell membrane or passing through it. It is assumed that the viruses which are independent of pH are also capable of using the endocytosis route (McClure et al., 1990).

When solving the problem of the invention, the starting premise was to make use of the mechanism used by certain viruses to penetrate into eucaryotic cells, in order to improve the transfer of nucleic acid complexes into the cell and thereby increase expression.

Attempts have been made to internalize proteins together with viruses into the cell (Otero and Carrasco, 1987). It was found that the permeability achieved in the cell by the virus is used to deliver macromolecules. The procedures taking place would appear to be fluid phase uptake mechanisms.

Using epidermal growth factor (EGF), conjugated to a toxin, it was found that this natural ligand, which is taken up into the cell by endocytosis after binding to its receptor, lands in the same endosome together with

the adenovirus, which is also taken up into the cell by receptor-mediated endocytosis, and is released from this endosome, again together with the virus, into the cytosol (FitzGerald et al., 1983).

It was found, surprisingly, that the presence of certain agents (e.g. viruses, virus components or other active substances), which exhibit the characteristics of certain viruses with regard to their mechanism to enter into eucaryotic cells, substantially increase the rate of expression of a nucleic acid imported into the cell as part of a complex. This finding was particularly surprising as the nucleic acid complexes taken up into the cell are very large.

The present invention thus relates to a composition for the transfection of higher eucaryotic cells with a complex of nucleic acid and a substance having an affinity for nucleic acid, which substance is optionally coupled with an internalizing factor. The composition is characterized in that it contains an agent which has the ability to be internalized into the cells which are being transfected, either per se or as a component of the nucleic acid complexes, and of releasing the contents of the endosomes, in which the complex is located after entering the cell, into the cytoplasm.

This agent is hereinafter referred to as "endosomolytic agent".

The ability of the endosomolytic agents to be taken up into the cells and to release the contents of the endosomes, in which they are located after entering the cell, into the cytoplasm, is hereinafter referred to as "uptake function". This uptake function comprises the ability to be internalized into the cell actively, via receptor-dependent endocytosis mechanisms, or passively, via the liquid phase or as a constituent of the nucleic acid complex, and the ability to break up endosomes, which is generally referred to as endosomolytic activity or endosomolysis.

In one embodiment of the invention the endosomclytic agent is a virus. In another embodiment the endosomolytic agent is a virus component. The virus or virus component employed in these embodiments of the invention is hereinafter referred to as "free" virus (component).

Within the scope of the present invention, the activity of an increasing dose of free adenovirus on the gene transfer capacity of a constant quantity of transferrin-polylysine conjugate in HeLa cells was investigated, using the luciferase gene as reporter gene. The augmentation in gene transfer brought about by the adenovirus reached a peak at $1x10^4$ virus particles per cell, a number which corresponds to the approximate number of adenovirus receptors per HeLa cell. The augmentation, up to 2000-fold, of luciferase expression compared with the expression achieved with the transferrin-polylysine conjugates alone, corresponded to the higher dose of virus. In another series of experiments, the capacity of limiting quantities of conjugate-DNA complexes was investigated in the presence of a constant dosage of free adenovirus. It was found that the uptake of adenoviruses into the cells augmented the gene transfer mediated by transferrin-polylysine over a wide range of DNA dosages. The maximum intensity of gene expression achieved by means of the conjugate-DNA complexes corresponded to the intensity achieved with 100 times less DNA when adenoviruses were used to increase the efficiency of transfection.

The effect of adenoviral infection on gene transfer was examined for both uncomplexed DNA and DNA that had been complexed with polylysine or transferrin-polylysine conjugates (Fig. 3A). By this analysis, adenoviral infection did not significantly augment transfer of naked, uncomplexed DNA during transfection. In marked contrast, transfer of DNA complexed to polylysine or transferrin-polylysine conjugates was augmented by

adenoviral infection. This effect was, however, much greater for the transferrin-polylysine conjugates. Since the polycation portion of the conjugate molecule not only serves to attach transferrin to DNA, but also effects significant structural changes in the DNA (Compacting into toroidal structures; Wagner et al., 1991a), these experiments could not initially differentiate whether the observed effect was on the basis of enhanced fluid-phase transport of the polycation-condensed DNA or virus-augmented delivery of receptor-bound conjugate-DNA complex. To distinguish between these possibilities, sequential binding experiments were performed (Fig. 3B). Binding of transferrin-polylysine-DNA or polylysine-DNA complexes at low temperature without internalization allowed removal of excess complex in the fluid phase prior to adenoviral infection (FitzGerald et al., 1983). When administered in this fashion, delivery of the receptor-bound transferrin-polylysine-DNA complexes was significantly augmented by the addition of adenoviral particles, whereas the polylysine-DNA complexes were not. Thus, it is the entry of DNA into the cell by the receptor-mediated endocytosis pathway which is specifically enhanced.

Next, analysis was made of the specific adenoviral function which brings about enhanced receptor-mediated gene transfer (Fig. 3C). Mild heat treatment of virus particles does not alter their ability to bind to target cell membranes but does affect their capacity to disrupt endosomes after internalization (Defer et al., 1990). Thus, the distinct effects of viral binding and viral entry into the cell could be separately evaluated on the basis of this finding. The tests carried out within the scope of this invention showed that heat inactivation of the adenoviruses completely abolished their ability to enhance receptor-mediated gene transfer via receptor-mediated endocytosis. This suggests that it is the

capacity of the adenoviruses to disrupt endosomes as part of their entry mechanism which specifically effects enhancement of gene delivery by transferrin-polylysine conjugates. The fact that a replication-defective virus strain could result in an increase in gene expression confirms the assumption that this phenomenon is not due to the replication function but due to the uptake function of the virion.

To rule out the possibility that the increase in gene expression can be ascribed to possible transactivation of the imported gene by the virus, experiments were carried out with a cell line which constitutively expresses the RSV-LTR luciferase gene: adenoviruses show no effects in this cell line, whereas in the parental cell line into which the gene had been introduced by means of transferrin-polylysine conjugates, there was a significant increase in gene expression. This finding demonstrates that the adenovirus influences events which take place before transcription and that its enhancing effect on gene transfer thus acts at the gene transfer level and not at the gene expression level (Fig. 5).

Investigations were also carried out within the scope of the invention to find out what effect adenoviruses have on gene transfer by means of transferrin-polylysine conjugates in selected cell lines. It was found that the presence of transferrin receptors on target cells is necessary, but not sufficient in every case, to permit gene transfer by transferrin-polylysine conjugates. Cell-specific factors relating to the fate of endosome-internalized conjugate-DNA complexes appear to be an extremely important determining factor in the levels of gene transfer achievable by this route. In this regard, selected cell lines were examined for both gene transfer by transferrin-polylysine conjugates and augmentation of gene transfer by adenoviruses (Fig. 4). Cells of a

cystic fibrosis cell line (CFT1) showed moderate levels of luciferase gene expression after treatment with transferrin-polylysine-DNA complexes. This level of expression was significantly augmented by treatment with the adenovirus d1312. In marked contrast, KB cells treated with the transferrin-polylysine-DNA complexes exhibited levels of luciferase gene expression barely above background levels, despite the presence of transferrin receptors. Treatment with adenovirus d1312, however, resulted in readily detectable luciferase activities in these cells. Treatment of HeLa cells with adenoviruses had a similar effect, although this effect was substantially stronger in these cells. Since HeLa cells and KB cells possess approximately the same number of receptors for the adenovirus, the difference in augmentation of the gene transfer may reflect the number of transferrin receptors characteristic of each cell type. However, in marked contrast to these results, the cell lines WI-38 and MRC-5, which are known to support adenoviral infection very poorly (Precious and Russell, 1985), showed very little augmentation with d1312 of the gene expression achieved by means of the conjugate-DNA complexes alone. Treatment with adenovirus, would therefore appear to augment gene transfer by means of conjugate-DNA complexes in those cases where the gene transfer is possible by receptor-mediated endocytosis, as in the case of CFT1 cells, and also in some instances where gene transfer by this route appears to be ineffective, as for HeLa and KB cells. The level of augmentation achieved varies significantly among different target cells, suggesting that this effect is a function of both the number of virus receptors, e.g. adenovirus receptors, of a certain cell type and also the number of transferrin receptors.

In case of the use of free virus, the substance having an affinity for nucleic acid, preferably an organic polycation, is preferably conjugated with an

internalizing factor. It has, however, been found, according to the invention, that under certain circumstances DNA complexed only with a substance having an affinity for nucleic acid, i.e. without internalizing factor, can be introduced into the cell in the presence of free virus. It was also found that, in some cell lines, the complexes consisting of nucleic acid and a substance having an affinity for nucleic acid can be introduced through the fluid phase if the concentration of the complexes is high enough. The experiments carried out within the scope of the present invention and previous ones showed that an essential element for the uptake capacity of the nucleic acid complexes is their compactness, which can be ascribed to the condensing of the nucleic acid by the substance having an affinity for nucleic acid. If the substance having an affinity for nucleic acid has sufficient capacity for binding to the cell surface in order to enter into the cell together with the virus, as well as being able to render the complex substantially electroneutral and condense the nucleic acid into a compact structure, there may not be a need to increase the entry capacity by covalently binding an internalizing factor to the substance having an affinity for nucleic acid in order to transfer the complex into the cell by receptor-mediated endocytosis. Many cells have a relatively high affinity for certain substances having an affinity for nucleic acid, so that the conjugates of nucleic acid and binding factor are taken up into the cell without the need for an internalizing factor. This is true, for example, of hepatocytes, which have been found within the scope of the present invention to take up DNA-polylysine complexes.

In a preferred embodiment of the invention, the endosomolytic agent is a virus which is bound to a substance having an affinity for nucleic acid and which has the ability to enter the cell as part of a

conjugate/nucleic acid complex and release the contents of the endosomes, in which the complex is located after entering the cell, into the cytoplasm.

In another preferred embodiment, the endosomolytic agent is a virus component which is bound to a substance having an affinity for nucleic acid and which has the ability to enter the cell as part of a conjugate/nucleic acid complex and release the contents of the endosomes, in which the complex is located after entering the cell, into the cytoplasm.

Viruses or virus components bound to the nucleic acid binding domain, irrespective of the type of binding, are hereinafter designated "viral conjugates".

The viral conjugates, which are also subject of the present invention, contain the virus or virus component as an integral part of their functional construct and combine the advantages of vector systems based on internalizing factor conjugates with the advantages which the viruses bring into these systems.

Furthermore, the viral conjugates according to these embodiments of the invention have the advantage that they circumvent the fundamental restriction inherent in the known bifunctional conjugate systems for gene transfer by receptor-mediated endocytosis, in that they have a specific mechanism which enables them to be released from the cell vesicle system. The viral conjugates according to the invention constitute a fundamental conceptual departure from the recombinant viral vectors, in that the foreign DNA which is to be transported is carried on the outside of the virion. Consequently, the viral conjugates according to the invention can transport very large gene constructs into the cell, with no restrictions as to the sequence.

The suitability of a virus, which is to be employed as free or bound virus or part of a virus as virus component within the scope of the present invention is defined by its uptake function. Suitable viruses are,

on the one hand, those which have the ability to enter into the cell by receptor-mediated endocytosis during transfection of the cells with the nucleic acid complex and bring about their release - and hence the release of the nucleic acid - from the endosome into the cytoplasm.

Without wishing to be tied to this theory, this mechanism could benefit the nucleic acid complexes transferred into the cell in so far as these complexes are conveyed together with the viruses from the endosomes into the cytoplasm, assuming that they arrive in the same endosomes as the viruses on being internalized. When the nucleic acid complexes contain the virus in bound form they benefit from the virus' endosomolytic activity and are conveyed from the endosomes into the cytoplasm. This avoids the fusion between endosomes and lysosomes and consequently the enzymatic degradation which normally takes place in these cell organelles.

For examples of viruses and higher eucaryotic cells into which they are capable of penetrating, reference in made to Fields and Knipe 1990, for example. The susceptibility of a given cell line to transformation by a virus used in the form of a free virus to facilitate entry of nucleic acid complexes into cells is dependent upon the presence and number of target cell surface receptors for the virus. With regard to the adenoviral cell surface receptor methods for determining its number on HeLa and KB cells are taught by Svensson, 1990, and Defer, 1990.

Viruses which are suitable for the composition according to the invention and whose uptake function, occurring at the start of infection, occurs by receptor-mediated endocytosis, include on the one hand viruses without a lipid coat such as adenovirus, poliovirus, rhinovirus, and on the other hand the enveloped viruses vesicular stomatitis virus, Semliki Forest virus, influenza virus; pH-dependent strains of Moloney virus

are also suitable. Particularly preferred viruses which may be used in the practice of the invention include Adenovirus subgroup C, type 5, Semliki Forest Virus, Vesicular Stomatitis Virus, Poliovirus, Rhinoviruses and Moloney Leukemia Virus.

The use of RNA viruses which have no reverse transcriptase in the present invention has the advantage that transfection in the presence of such a virus does not result in generation of viral DNA in the transfected cell. In the present invention, Rhinovirus HRV2, a representative of the Picornavirus group, was shown to increase expression of a reporter gene. The efficacy of the Rhinovirus was demonstrated both in free form and in the form of virus conjugates.

Within the scope of the present invention, the term viruses - provided that they are taken up into the cell and release the contents of the endosomes in which they arrive - includes in addition to the wild types, mutants which have lost certain functions of the wild type, other than their uptake function, especially their ability to replicate, as a result of one or more mutations.

Mutants are produced by conventional mutagenesis processes by mutations or deletions in virus-protein regions which are responsible for the replicative functions, are not necessary for the uptake function and which may be complemented by a packaging line. These include, e.g. in the case of adenovirus, ts-mutants (temperature sensitive mutants), E1A- and E1B-mutants, mutants which exhibit mutations in MLP-driven genes (Berkner, 1988) and mutants which exhibit mutations in the regions of certain capsid proteins. Virus strains which have corresponding natural mutations are also suitable. The ability of viruses to replicate can be investigated, for example, using plaque assays known from the literature, in which cell cultures are covered with suspensions of various virus concentrations and the

number of lysed cells which is visible by means of plaques is recorded (Dulbecco, 1980).

Other viruses which may be suitable for use within the scope of the invention include so-called defective viruses, i.e. viruses which lack the function necessary for autonomous virus replication in one or more genes, for which they require helper viruses. Examples of this category are DI-particles (defective interfering particles) which are derived from the infectious standard virus, have the same structural proteins as the standard virus, have mutations and require the standard virus as a helper virus for replication (Huang, 1987; Holland, 1990). Examples of this group also include the satellite viruses (Holland, 1990). Another group is the class of parvoviruses called adeno-associated virus (Berns, K.I., 1990). Since the entry cycles of many viruses in the cells are not completely characterized, it is likely that there will be other viruses that will exhibit the endosomolytic activity required for their suitability in the present invention.

Also suitable within the scope of this invention may be attenuated live vaccines (Ginsberg, 1980) or vaccination strains.

The term viruses within the scope of the present invention also includes inactivated viruses, e.g. viruses inactivated by chemical treatment such as treatment with formaldehyde, by UV-radiation, by chemical treatment combined with UV-radiation, e.g. psoralen/UV-radiation or bromodeoxyuridine treatment, by gamma-radiation or by neutron bombardment. Inactivated viruses, e.g. such as are also used for vaccines, may be prepared by standard methods known from the literature (Davis and Dulbecco, 1980, Hearst and Thiry, 1977) and tested for their suitability to increase the transfer of DNA complexes. In experiments carried out within the scope of the present invention, adenovirus preparations were inactivated using a conventional UV sterilization

lamp or with formaldehyde. It was surprisingly found that the degree of inactivation of the viruses was substantially greater than the reduction in the gene transfer effect, which was achieved when adenovirus was added to the transfection medium. Experiments carried out with preparations of psoralen/UV-inactivated biotinylated adenovirus, which was coupled with streptavidin-coupled polylysine, also showed that as a result of the inactivation the virus titer decreased considerably more sharply than the gene transfer capacicy. This is a clear indication that mechanisms which are connected with the normal infection mechanism in the active virus can be destroyed without destroying the effect which is essential for gene transfer.

The term "virus components" denotes parts of viruses, e.g. the protein part freed from nucleic acid (the empty virus capsid, which may be produced by recombinant methods, e.g. Ansardi et al., 1991; Urakawa et al., 1989), proteins obtained by fractionation or peptides which have the endosomolytic functions of the intact virus essential for the uptake function. These virus components may be produced synthetically, depending on their size either by peptide synthesis or by recombinant methods. In the present invention adenovirus proteins conjugated via biotin/streptavidin to polylysine were demonstrated to enhance gene transfer. Examples of fragments of proteins from viruses other than adenovirus, which are essential for internalization, include influenza virus hemagglutinin (HA). The N-terminal sequence of the influenza virus hemagglutinin HA2 subunit is responsible for releasing the virus from the endosome. It has been shown that peptides consisting of 20 amino acids of this sequence are capable of fusing lipid membranes and partly breaking them open or destroying them (Wharton et al., 1988). In the present invention, authentic and modified influenza peptides were successfully employed in various

embodiments. Another example are coat proteins of retroviruses, e.g. HIV gp41 (Rafalski et al., 1990) or parts of these virus proteins.

The use of viruses which have the ability per se to enter cells and thus function as internalization factors, is but one aspect of the present invention.

Viruses or virus components which themselves do not bring the capacity to bind to the cell and enter into it, are preferably used as viral conjugates as defined above. Coupling to a DNA binding domain, e.g. a polycation, ensures that the virus or virus component acquires a high affinity for DNA molecules and is thus complexed to it and transported into the cell as a component of the DNA complex, which also contains a conjugate of internalizing factor and DNA binding domain. In addition to the transfer effect thus achieved, binding of the virus or virus component to a nucleic acid binding domain may also result in an improvement in its endosomolytic properties.

By choosing other internalization factors, practically any higher eucaryotic cell may be transfected with the compositions of the present invention.

One can determine with a simple screening assay whether a given virus or virus component has an uptake function as defined in the invention and is thus suitable for augmenting gene transfer. In this assay, e.g. for testing a virus for its applicability as free virus, the target cells are contacted with a DNA complex in the presence or absence of the virus. The amount of DNA complex released into the cytoplasm can then be easily determined by detection of a marker gene product, e.g. luciferase. If the presence of the virus causes the DNA complex to be taken up and released into the cytoplasm at a greater level than without the virus, this may be attributed to the uptake function of the virus. It is also possible to compare the level of

uptake with the test virus when compared to another virus known to have a suitable uptake function, e.g. adenovirus subgroup C, type 5. Tests of this kind may also be applied to viral conjugates, whilst additional parameters such as various internalizing factor conjugates in varying amounts may be subject to such tests. Furthermore, a person skilled in the art can easily apply assays of this kind, optionally in combination with other tests, e.g. liposome leakage assays, for testing virus components or other agents with potential endosomolytic activity for their ability to enhance gene expression.

When intact viruses are used, tests are carried out, preferably parallel to the preliminary tests investigating the virus for its ability to augment gene transfer, to see whether the virus is capable of replicating. The investigation for ability to replicate is carried out by using plaque assays (see above) in the case of cytopathic viruses or in the case of viruses which significantly impair the growth of the host cells. For other viruses, detection methods specific to the virus in question are used, e.g. the hemagglutination test or chemico-physical methods, e.g. using an electron microscope.

Within the scope of this invention, the preferred viruses, in particul those which are applied as free viruses, are those which can be produced in a high titer, which are stable, have low pathogenicity in their native state and in which a targeted elimination of the ability to replicate is possible, especially adenoviruses. If a specific cell population is to be transfected, viruses which specifically infect this cell population are preferred. If the transfection is intended to target different cell types, viruses which are infectious for a wide range of cell types may be used.

The requirements are that the virus preparation

should be of the greatest possible purity and that a stabilizing buffer should be used which is matched to the particular virus.

In any case, for therapeutic use of the invention in vivo only those viruses or virus components may be used in which the safety risks are minimized as far as possible, particularly the risk of replication of the virus in the target cell and recombination of virus DNA with host DNA.

Advantageously, the entry mechanism of viruses which infect animals other than humans may be used to enhance the uptake and release of DNA into higher eucaryotic cells, especially of humans, so long as the virus exhibits endosome disruption activity in the higher eucaryotic cells. Members of the adenovirus family have been isolated from avian species, from amphibians and from a variety of other animals (see, for example, Laver et al., 1971;, Bragg et al., 1991; Akopian et al., 1991; Takase et al., 1990; Khang and Nagaraji 1989; and Reece et al., 1987). Amphibian, avian, bovine, canine, murine, ovine, porcine and simian adenoviruses, as well as human adenoviruses, are available from the American Type Culture Collection, Rockville, Maryland (See the American Type Culture Collection Catalogue of Animal Viruses and Antisera, Chlamydae and Rickettsiae, Sixth Edition, 1990, C. Buck and G. Paulino eds., pp. 1-17).

Possible advantages of using a virus, e.g. an adenovirus, from a distant species might be a reduced toxicity in the target cells (e.g. the chicken or frog adenovirus would not be expected to replicate or initiate early gene expression in mammalian cells), a reduced hazard to the investigator preparing the distant adenovirus, compared to the human adenovirus, and reduced interference from antibodies against human or murine adenovirus. The absence of interference by the human or murine antibodies is particularly important

when the viruses are employed in gene therapy in humans and mice.

The chicken adenovirus CELO (chick embryo lethal orphan virus) shows no reactivity to antibodies that recognize the major group epitopes of the adenoviruses infecting mammalian cells. Moreover, CELO virus may be grown in embryonated eggs to give high levels of virus (0.5 mg/egg; Laver et al., 1971). As shown in the Examples, CELO-polylysine conjugates augment DNA delivery to HeLa cells at levels comparable to the human adenovirus dl312. Thus, the use of CELO conjugates to augment DNA delivery holds great promise in human gene therapy experiments.

Viruses of distant species are preferably used as constituents of viral conjugates in combination complexes, as herein defined.

In conjugates of the invention which contain a virus, binding of the virus to the nucleic acid binding domain may be covalent or non-covalent, e.g. a biotin-streptavidin bridge or an ionic binding if the virus has areas on its surface proteins which are acidic and therefore can bind to a polycation.

In experiments of the present invention, complexes were formed under conditions which allow ionic interaction between adenovirus and polylysine before complexing with DNA. Control experiments were conducted under conditions where polylysine is first neutralized with DNA and is therefore not free to bind the adenovirus. The complexes with ionically bound adenovirus were superior in these experiments.

Examples of virus components in the scope of the invention with endosomolytic activity are the empty virus capsids or viral peptides. Binding of the virus component to the nucleic acid binding domain may be covalent, e.g. by chemically coupling the viral peptide with polylysine, or non-covalent, e.g. ionic in cases where the virus component has acid residues to bind to a

polycation.

The ratio of virus or virus component to the substance having affinity to nucleic acid may be varied. In the case of influenza haemagglutinin peptide-polylysine conjugate it was found in the present invention that gene transfer can be augmented to a greater extent when the content of viral peptide in the conjugates is higher.

In another aspect the present invention relates to methods of preparing the conjugates according to the invention from virus (component) and a substance having an affinity for nucleic acid.

The viral conjugates may be prepared (like the internalizing factor-polycation conjugates) by coupling the components or, if the virus component and polycation are polypeptides, by the recombinant method; with regard to methods of preparation reference is made to the disclosure of EP 388 758.

Binding of virus or viral proteins or peptides, respectively, with polyamine compounds by the chemical method can be effected in the manner which is already known for the coupling of peptides and if necessary the individual components may be provided with linker substances before the coupling reaction (this measure is necessary if there is no functional group available which is suitable for the coupling, e.g. a mercapto or alcohol group). The linker substances are bifunctional compounds which are reacted first with functional groups of the individual components, after which the modified individual components are coupled.

Coupling may be carried out by means of

a) Disulphide bridges, which can be cleaved again under reducing conditions (e.g. when using succinimidyl-pyridyldithiopropionate (Jung et al., 1981).

b) Compounds which are substantially stable under

biological conditions (e.g. thicethers by reacting maleimido linkers with sulfhydryl groups of the linker bound to the second component).

c) Bridges which are unstable under biological conditions, e.g. ester bonds, or acetal or ketal bonds which are unstable under slightly acidic conditions.

In experiments carried out within the scope of the present invention, endosomolytic influenza-hemagglutinin HA2-peptides were coupled with polylysine by the chemical method using succinimidylpyridyldithio-propionate (SPDP). It was shown that the modification of the peptide with polylysine increases the endosomolytic activity. Transfection experiments showed that the efficiency of gene transfer mediated by transferrin-polylysine is substantially increased if the influenza peptide-polylysine conjugates are present together with transferrin-polylysine in the DNA complex.

Moreover, within the scope of the present invention, adenovirus was bound to polylysine by various different methods. One way of conjugating the virus with polylysine was effected in a similar manner to the production of transferrin-polylysine conjugates (Wagner et al., 1990) after modification of the defective adenovirus dl312 using a heterobifunctional reagent. Unbound polylysine was removed by centrifuging. The DNA binding capacity was demonstrated in a binding experiment using radioactively labelled DNA. In K562 cells in the absence of chloroquine, substantially higher gene transfer was found with complexes consisting of DNA, adenovirus-polylysine and transferrin-polylysine, than with unmodified adenovirus which is not bound to the DNA. It was also found that significant gene expression occurred with only 0.0003 µg of DNA in 5×10^5 HeLa cells using polylysine-modified adenovirus.

If the virus or virus component contains suitable carbohydrate chains, they may be linked to the substance

having an affinity for nucleic acid via one or more carbohydrate chains of the glycoprotein.

A suitable method of preparing glycoprotein-polycation conjugates is disclosed in German patent application P 41 15 038.4; it has recently been described by Wagner et al., 1991b.

Another preferred method of preparing the viral conjugates of the invention is by enzymatic coupling of the virus or virus component to a substance having an affinity for nucleic acid, more particularly a polyamine, by means of a transglutaminase.

The category of transglutaminases comprises a number of different enzymes which occur inter alia in the epidermis (epidermal transglutaminase), in the blood (Factor XIII) and in the cells of various tissues (tissue transglutaminase) (Folk, 1985). Transglutaminases catalyze the formation of ϵ-(γ-glutamyl)lysine bonds in the presence of Ca++ and with cleaving of NH_3. The prerequisite for this is that corresponding glutamines and lysines should be present in proteins, capable of being reacted by the enzyme. Apart from the ϵ-amino group of lysine, (poly)amines such as ethanolamine, putrescine, spermine or spermidine may also be used as substrate (Clarke et al., 1959). At present it is not yet clear what the critical factors are which determine whether a glutamine or lysine of a protein or a polyamine can be reacted by the enzyme. What is known is that polyamines can be bound by means of transglutaminase to numerous cell proteins such as cytokeratins (Zatloukal et al., 1989), tubulin, cell membrane proteins and also surface proteins of influenza viruses (Iwanij, 1977).

Within the scope of the present invention it has been shown that polylysine can be coupled to adenoviruses by means of transglutaminase. It was found that coupling can be carried out in the presence of glycerol. This has the advantage that a virus

preparation, e.g. an adenovirus preparation which contains glycerol as stabilizing agent in the buffer, can be used directly for coupling. Using adenovirus-polylysine conjugates which were complexed with plasmid-DNA together with transferrin-polylysine conjugates, it was possible to achieve many times greater gene expression than with transferrin-polylysine conjugates in the presence of non-polylysine-coupled adenovirus.

Another method of preparing the conjugates according to the invention which is preferred within the scope of the invention consists in coupling the virus or virus component to the polycation via a biotin-protein bridge, preferably a biotin-streptavidin bridge.

The known strong association of biotin with streptavidin or avidin (Wilchek et al., 1988) was used for coupling adenovirus to polylysine by modifying adenovirus with biotin and chemically conjugating streptavidin to polylysine in a similar manner to the production of transferrin-polylysine conjugates (Wagner et al., 1990). Complexes consisting of DNA and streptavidin-polylysine, to which the biotin-modified virus is bound, and optionally non-covalently bound polylysine, having a very high transfection efficiency, even at lower concentrations of DNA. Particularly efficient complexes are formed if the biotin-modified virus is first bound to streptavidin-polylysine and the binding to DNA only occurs in a second step.

If desired, the binding to biotin may also be effected by means of avidin.

It is also possible to establish the bond between the virus (component) and polylysine by biotinylating the virus, on the one hand, and conjugating an anti-biotin antibody with polylysine, on the other hand, and establishing the bond between the virus and the polylysine by means of the biotin/antibody bond, using standard commercially available polyclonal or monoclonal anti-biotin antibodies.

Binding between the virus and polylysine may also be achieved by coupling polylysine with a lectin which has an affinity for a virus surface glycoprotein, the bonding in such a conjugate being effected by means of the bond between the lectin and the glycoprotein. If the virus does not have any suitable carbohydrate side chains itself, it may be suitably modified.

A virus may also be bound to a substance having an affinity for nucleic acid by first being modified on the surface with an antigen alien to the virus (e.g. digoxigenin DIG, obtainable from Boehringer Mannheim; or with biotin) and establishing the connection between the modified virus and the substance having an affinity for nucleic acid via an antibody which binds to this antigen. The particular method which will be used to produce the conjugates according to the invention depends on various criteria. Thus, for example, coupling by means of biotin is the least specific and therefore most widely applicable method, while the biotin-mediated binding constitutes a very strong non-covalent bonding. The enzymatic reaction with transglutaminase has the advantage that it can also be carried out on a very small scale. Chemical coupling is generally used when larger quantities of conjugate are to be synthesized and this method is generally also the best when coupling virus proteins or peptides. If inactivated viruses are used, the inactivation is generally carried out before the coupling, provided that the coupling is not affected by the inactivation.

If a virus, e.g. adenovirus, or an endosomolytic component thereof, has binding domains accessible, e.g. acidic domains for binding to a polycation, binding of the virus (component) to the polycation may also be ionic. In this case, the positive charges of the polycation, which is optionally conjugated with an internalizing factor, are partially neutralized by the acidic domain of the virus (component), the remainder of

the positive charges will be essentially neutralized by the nucleic acid.

If the substance having an affinity for nucleic acid is an intercalating substance, it is modified with a linker which is suitable for the particular coupling of virus (component), e.g. for coupling with transglutaminase it is modified with spermine or with a bifunctional group competent for chemical coupling, e.g. an active ester.

The ratio of virus (component): nucleic acid binding substances may vary; it is usually established empirically, e.g. by conjugating a constant amount of virus (component) with different amounts of polylysine and selecting the optimal conjugate for the transfection.

In another embodiment of the invention, the virus component, e.g. an endosomolytic viral peptide, may be modified in order to bind direct to DNA. To this end the peptide itself may contain a DNA binding domain which is obtainable by producing the peptide by means of peptide synthesis and providing a stretch of positively charged aminoacids, preferably by extending the peptide, most preferably at the C-terminus.

In another embodiment of the invention the endosomolytic agent is a non-viral, optionally synthetic peptide. A peptide of this type is preferably contained in the composition according to the invention in such a way that it is ionically bound to the substance with affinity to nucleic acid, e.g. to polylysine in the case of DNA-internalizing factor-polylysine complexes. Thereby incorporation of the endosomolytic peptide into the nucleic acid complexes is accomplished by binding the peptide via its acidic amino acid residues to the positively charged nucleic acid binding domain, preferably polylysine.

Depending on the chemical structure of the peptide, in particular with regard to its end group, binding to

polylysine may also be accomplished by the methods described herein for linking peptides to polylysine. To this end, if a naturally occurring peptide is employed, it may be modified with a suitable terminal amino acid as a handle for conjugation.

Another way of incorporating non-viral endosomolytic peptides into the nucleic acid complexes is to provide them with sequences which bind to DNA. The location of such a sequence has to be such that it does not interfere with the peptide's endosomolytic activity. Therefore, for example, peptides whose N-terminus is responsible for this acitivity, are extended by DNA binding sequences at the C-terminus. Extensions of this kind may be homologous or heterologous cationic oligopeptides, e.g. an oligo-lysine tail, or a natural DNA binding domain, e.g a peptide derived from a histone. Preferably these DNA binding sequences as integral part of the endosomolytic peptide comprise approximately 10 to 40 amino acids. This embodiment of the invention offers the possibility of a higher ratio of endosomolytic sequence to DNA binding sequence than in peptide conjugates which contain larger portions of polycations in order to achieve a higher efficiency of the complexes.

The non-viral endosomolytic peptides should fulfil the following requirements:

With regard to endosomolytic acitivity the leakage of lipid membranes achieved by the peptide should preferably be higher at low pH (5-6) than at pH 7. Furthermore, the disrupted areas of the membrane should be large enough to allow passage of large DNA complexes (small pores are not sufficient). In order to determine whether a peptide fulfils these requiments, <u>in vitro</u> tests can be carried out by applying the peptides in free or bound form and/or incorporated in a DNA complex. Such assays may comprise liposome leakage assays or

erythrocyte leakage assays and cell culture experiments, in which augmentation of gene expression is determined. Tests of this type are described in the Examples. The optimal amount of peptide can be determined in preliminary titrations by assaying the resulting gene transfer efficiency. It has to be born in mind that efficiency of various peptides and optimal composition of the complex may depend on cell type.

Membrane disruptive peptides in general contain amphipathic sequences, namely a hydrophobic face that may interact with the lipid membrane, and a hydrophilic face that stabilizes the aqueous phase at the membrane disruption site.

There are several examples of membrane-disruptive peptides in nature, usually small peptides or peptide domains of large polypeptides. Such peptides may be classified according to their function in the natural context, namely either in membrane disrupting peptides (e.g. peptides of naked viruses) and/or membrane fusing peptides (e.g. enveloped viruses). For the purpose of endosome disruption in the context of synthetic peptides both classes of peptide sequences may be useful. Most of the natural peptides are able to form amphipathic α-helices.

pH-specificity may be achieved by incorporation of acidic residues onto the hydrophilic face of a putative amphipathic α-helix in such a way that the helix can form only at acidic pH, but not at neutral pH where charge repulsion between the negatively charged acidic residues prevents helix formation. This property is also found with naturally occurring sequences (e.g. influenza HA-2 N-terminus).

A completely synthetic, amphipathic peptide with pH-specific membrane-disruption properties has been described by Subbarao et al., 1987 and by Parente et al., 1990. This peptide (in free form) was shown to form only small pores in membranes, allowing only the

release of small compounds (Parente et al., 1990).

According to the embodiment of the invention which makes use of non-viral, optionally synthetic peptides, usually the following steps are taken: an amphipathic peptide sequence is selected from the groups of naturally occuring or artificial peptides. Peptides of this kind are known in the art, a survey of examples is given in Table 2. If necessary, acidic residues (Glu, Asp) are introduced to make the peptide's membrane disrupting activity more pH-specific (e.g. the double acid mutant of the influenza hemagglutinin peptide according to Example 37, designated p50). If necessary, acidic residues may also be introduced in order to facilitate binding of the peptide to polylysine. One way to provide for such a polycation binding domain may be to introduce C-terminal acidic extensions, e.g. an oligo-Glu-tail.

Endosomolytic peptides suitable for the present invention may also be obtained by fusing naturally occurring and artificial sequences. In the present invention experiments were conducted with various peptides which were derived from the synthetic peptide GALA described by Parente et al., 1990. Some of the derivatives employed in the experiments of the present invention were obtained by combining the peptide GALA or modifications thereof with sequences of the influenza peptide or modifications thereof, e.g. the peptides designated EALA-Inf and EALA-P50 according to Example 33.

The length of the peptide sequence may be critical with regard to the stability of the amphipathic helix; an increase of stability of short domains derived from natural proteins, which lack the stabilizing protein context, may be achieved by elongation of the helix.

In order to increase the endosomolytic activity of the peptides, homodimers, heterodimers or oligomers may be formed; it has been shown in the experiments of the

present invention that a P50 dimer has a much higher activity than the monomer.

The present inventors have shown the effect of synthetic peptides on DNA uptake mediated by transferrin-polylysine conjugates. Various different peptides were synthesized, their liposome and erythrocyte leakage capacity assayed and their effect on luciferase expression in TIB 73 cells and in NIH 3T3 cells tested.

In another embodiment of the invention, the endosomolytic agent may be a non-peptidic amphipathic substance. The requirements such a substance must fulfil to be suitable for the present invention are essentially the same as for the amphipathic peptides, namely ability to be incorporated into the nucleic acid complexes, pH specificity, etc.

In another aspect the invention relates to complexes which are taken up into higher eucaryotic cells, containing nucleic acid and a conjugate which has the ability to form a complex with nucleic acid, for introducing nucleic acid into higher eucaryotic cells. The complexes are characterized in that the conjugate consists of a substance having an affinity for nucleic acid and an endosomolytic agent which is bound to the substance having an affinity for nucleic acid and has the ability of being internalized into the cell as part of a conjugate/nucleic acid complex and of releasing the contents of the endosomes, in which the complex is located after entering the cell, into the cytoplasm.

The nucleic acid complexes used within the scope of the invention are preferably those wherein the nucleic acid is complexed with a substance having an affinity for nucleic acid in such a way that the complexes are substantially electroneutral.

In a preferred embodiment of the invention, the endosomolytic agent is a virus or a virus component covalently bound to a polycation.

Within the scope of the present invention, the endosomolytic conjugates also encompass - in addition to conjugates in which endosomolytic agents are ionically bound to a DNA binding domain - endosomolytic agents which bind to DNA direct, e.g. via their basic extension, although "conjugates" of this kind are strictly speaking not obtained by conjugation, i.e. by binding two compounds to each other. The function of endosomolyic agents of this type as components of the composition according to the invention is independent of whether they were synthezised by conjugation of an endosomolytic agent and a DNA binding domain or whether a DNA binding domain was originally present in the endosomolytic agent.

In another preferred embodiment of the invention the complexes contain, in addition to the endosomolytic conjugate, another conjugate in which a substance having an affinity for nucleic acid, in case of an endosomolytic polycation conjugate generally the same polycation as in the conjugate, is conjugated to an internalizing factor having an affinity for the target cell. This embodiment of the invention is used particularly when the target cell has no or few receptors for the virus employed as part of the endosomolytic conjugate. Another application of this embodiment of the invention is when a virus component, e.g. a naturally occurring, optionally modified peptide, a non-viral, optionally synthetic endosomolytic peptide or a virus from a distant species are employed, which do not have the ability to penetrate by themselves into the cells which are to be transfected. In the presence of an additional internalizing factor-binding factor conjugate, the endosomolytic conjugates profit from the internalizing ability of the second conjugate, by being complexed to the nucleic acid together with the second conjugate and being taken up into the cell as part of the resulting complex which in the following is referred

to as "combination complex" or "ternary complex". Without being pinned down to this theory, the combination complexes are taken up by cells either by binding to the surface receptor which is specific to the internalizing factor or, if a virus or virus component is used, by binding to the virus receptor or by binding to both receptors and internalized by receptor-mediated endocytosis. When the endosomolytic agents are released from the endosomes the DNA contained in the complexes is also released into the cytoplasm and thereby escapes the lysosomal degradation.

In the experiments of the present invention with HeLa cells, nearly all the cells could be transfected with free adenovirus. The efficacy for hepatocytes could be still further improved when using ternary DNA complexes in which the reporter DNA is complexed to polylysine-transferrin conjugates and linked to adenovirus. Here, co-localization of the endosomolytic virus and the ligand/receptor complex in the endosome is guaranteed yielding transfection in virtually all cells for a variety of cells such as BNL.CL2 and HepG2 cells. Such a situation might be approximated in the experiments where ternary DNA complexes containing transferrin gained access to K562 cells in the main via the transferrin receptor rather than the adenovirus receptor.

Unexpectedly, ternary complexes transferred DNA even in very small amounts. Thus at an input of 30 pg DNA/3 x 10^5 cells, 1.8 x 10^4 light units (resulting from expression of a luciferase encoding plasmid) are obtained. At this input there are as little as 60 DNA molecules and 1 PFU (plaque forming unit) of virus per cell. This has to be compared to the less efficient calcium precipitation protocol which uses 2 x 10^5 DNA molecules per cell (Sambrook et al, 1989). Thus, the present invention represents a significant advance in the art since it allows for the efficient transformation

of higher eucaryotic cells with very small amounts of DNA.

The presence of viruses, virus components or non-viral endosomolytic agents in the DNA complexes as constituents of endosomolytic conjugates has the following advantages:

1) Broader applicability of the gene transfer technology with nucleic acid complexes, since the endosomolytic agent itself, in particular in cases where a virus or virus component is employed, may constitute the internalizing factor or may also be complexed to the DNA together with another internalizing factor (e.g. transferrin or asialofetuin etc.). In this way it is possible to make use of the positive effect of the viruses even for cells which do not have any receptor for the virus in question.

2) Improvement in the efficiency of gene transfer, since the binding of the endosomolytic conjugates to the DNA ensures that they are jointly taken up into the cells. The coordinated uptake and release of viruses and DNA also gives rise to the possibility of a reduction in the quantity of DNA and viruses required for efficient gene transfer, which is of particular importance for use in vivo.

The term "internalizing factor" for the purposes of the present invention refers to ligands or fragments thereof which, after binding to the cell are internalized by endocytosis, preferably receptor-mediated endocytosis, or factors, the binding or internalizing of which is carried out by fusion with elements of the cell membrane.

Suitable internalizing factors include the ligands transferrin (Klausner et al., 1983), conalbumin (Sennett et al., 1981), asialoglycoproteins (such as asialotransferrin, asialorosomucoid or asialofetuin)

(Ashwell et al., 1982), lectins (Goldstein et al., 1980, and Shardon, 1987) or substances which contain galactose and are internalized by the asialoglycoprotein receptor; mannosylated glycoproteins (Stahl et al., 1987), lysosomal enzymes (Sly et al., 1982), LDL (Goldstein et al., 1982), modified LDL (Goldstein et al., 1979), lipoproteins which are taken up into the cells via receptors (apo B100/LDL); viral proteins such as the HIV protein gp120; antibodies (Mellman et al., 1984; Kuhn et al., 1982, Abrahamson et al., 1982), or fragments thereof against cell surface antigens, e.g. anti-CD4, anti-CD7; cytokines such as interleukin-1 (Mizel et al., 1987), Interleukin 2 (Smith et al., 1985), TNF (Imamura et al., 1987), interferon (Anderson et al., 1982); CSF (colony-stimulating factor) (Walker et al., 1987); factors and growth factors such as insulin (Marshall, 1985), EGF (epidermal growth factor) (Carpenter, 1984); PDGF (platelet-derived growth factor) (Heldin et al., 1982), TGFβ (transforming growth factor β) (Massague et al., 1986), nerve growth factor (Hosang et al., 1987), insulin-like growth factor I (Schalch et al., 1986), LH, FSH, (Ascoli et al., 1978), growth hormone (Hizuka et al., 1981), prolactin (Posner et al., 1982), glucagon (Asada-Kubota et al., 1983), thyroid hormones (Cheng et al., 1980); α-2-macroglobulin protease (Kaplan et al., 1979); and "disarmed" toxins. Further examples are immunoglobulins or fragments therof as ligands for the Fc receptor or anti-immunoglobulin antibodies, which bind to SIgs (surface immunoglobulins). The ligands may be of natural or synthetic origin, (see, Trends Pharmacol. Sci., 1989, and the references cited therein).

The following are essential requirements for the suitability of such factors according to the present invention,

a) that they can be internalized by the specific cell type into which the nucleic acid is to be

introduced and their ability to be internalized is not affected or only slightly affected if they are conjugated with the binding factor, and

b) that, within the scope of this property, they are capable of carrying nucleic acid "piggyback" into the cell by the route they use.

In the experiments carried out according to the invention, the wide range of uses of the invention regarding the internalizing factor, or additional internalizing factor in the combination complexes, respectively, is demonstrated by means of human and mouse transferrin-polylysine-conjugates, asialofetuin-polylysine-conjugates, galactose-polylysine-conjugates, wheat germ agglutinin-polylysine-conjugates, the T-cell-specific gp120-pL and antiCD7-pL conjugates, LDL-pL conjugates, Ig-pL and anti-Ig-pL conjugates and by means of DNA- polylysine-complexes which do not contain any internalizing factor. Moreover, the performance of the virus conjugates according to the invention was demonstrated by means of complexes of DNA and polylysine-conjugated virus (or virus component) which contained no additional internalizing factor-binding factor conjugate.

Specifically preliminary tests can be carried out to determine whether, if the endosomolytic agent is a free virus, the use of an internalizing factor, or if the endosomolytic agent is a virus or a virus component or a non-viral peptide which is part of an endosomolytic conjugate, an "additional" internalizing factor permits or improves the uptake of nucleic acid complexes. These tests comprise parallel transfections with nucleic acid complexes, firstly without (additional) internalizing factor, e.g. in case of virus conjugates with complexes consisting of nucleic acid and virus conjugate, and secondly with complexes in which the nucleic acid is complexed with another conjugate containing an

additional internalizing factor for which the target cells have a receptor.

If an internalizing factor is used, or if an additional internalizing factor is used, i.e. a combination complex is applied, it is defined particularly by the target cells, e.g. by specific surface antigens or receptors specific to a cell type which thus permit the targeted transfer of nucleic acid into this cell type.

Substances with an affinity for nucleic acid which may be used according to the invention include, for example, homologous organic polycations such as polylysine, polyarginine, polyornithine or heterologous polycations having two or more different positively charged amino acids, these polycations possibly having different chain lengths, and also non-peptidic synthetic polycations such as polyethyleneimine. Other substances with an affinity for nucleic acid which are suitable are natural DNA-binding proteins of a polycationic nature such as histones or protamines or analogues or fragments thereof, as well as spermine or spermidines.

The length of the polycation is not critical, as long as the complexes are substantially electroneutral. The preferred range of polylysine chain lengths is from about 20 to about 1000 lysine monomers. However, for a given length of DNA, there is no critical length of the polycation. Where the DNA consists of 6,000 bp and 12,000 negative charges, the amount of polycation per mole DNA may be, e.g.:

60 moles of polylysine 200

30 moles of polylysine 400; or

120 moles of polylysine 100, etc.

One of ordinary skill in the art can select other combinations of polycation length and molar amount with no more than routine experimentation.

Other suitable substances with an affinity for nucleic acid as part of the conjugates are intercalating

substances such as ethidium dimers, acridine or intercalating peptides, containing tryptophan and/or tyrosine and/or phenylalanine.

As for the qualitative composition of the nucleic acid complexes, generally the nucleic acid to be transferred into the cell is determined first. The nucleic acid is defined primarily by the biological effect which is to be achieved in the cell and, in the case of use for gene therapy, by the gene or gene section which is to be expressed, e.g. for the purpose of replacing a defective gene, or by the target sequence of a gene which is to be inhibited. The nucleic acids to be transported into the cell may be DNAs or RNAs, while there are no restrictions imposed on the nucleotide sequence.

If the invention is applied on tumor cells in order to use them as a cancer vaccine, the DNA to be introduced into the cell preferably codes for an immmune modulating substance, e.g. a cytokine like IL-2, IL-4, IFN-gamma, TNF-α. Combinations of cytokine encoding DNAs may be particularly useful, e.g. IL-2 and IFN-gamma. Another useful gene to be introduced into tumor cells may be the multi drug resistance gene (mdr). In the present invention transferrin-polylysine and low density lipoprotein conjugates have successfully been used together with adenovirus conjugates for the transfection of tumour cells (melanoma cells). Depending on the specific application, preliminary tests can be used to determine which ligand is suitable for the type of tumour cell in the specific case in question.

It is also possible to introduce two or more different nucleic acid sequences into the cell, e.g. a plasmid containing cDNAs coding for two different proteins under the control of suitable regulatory sequences or two different plasmid constructs containing different cDNAs.

Therapeutically effective inhibiting nucleic acids for transfer into the cells in order to inhibit specific gene sequences include gene constructs from which antisense-RNA or ribozymes are transcribed. Furthermore, it is also possible to introduce oligonucleotides, e.g. antisense oligonucleotides, into the cell. Antisense oligonucleotides comprise preferably 15 nucleotides or more. Optionally, the oligonucleotides may be multimerized. When ribozymes are to be introduced into the cell, they are preferably introduced as part of a gene construct which comprises stabilizing gene elements, e.g. tRNA gene elements. Gene constructs of this type are disclosed in EP A 0 387 775. Inhibitory nucleic acids and the mechanisms of activity thereof are familiar to those skilled in the art; reference is made in this respect to the summarising articles by Hélène and Toulme, 1990, and Takayama and Inouye, 1990, as well as the references mentioned therein.

Apart from nucleic acid molecules which inhibit genes, e.g. viral genes, due to their complementarity, genes with a different mode of inhibitory action may be employed. Examples are genes coding for viral proteins which have so-called trans-dominant mutations (Herskowitz, 1987). Expression of the genes in the cell yields proteins which dominate the corresponding wildtype protein and thus protect the cell, which acquires "cellular immunity", by inhibiting viral replication. Suitable are trans-dominant mutations of viral proteins which are required for replication and expression, e.g. Gag-, Tat and Rev mutants which were shown to inhibit HIV replication (Trono et al., 1989; Green et al., 1989; Malim et al., 1989).

Another mechanism of achieving intracellular immunity involves expression of RNA molecules containing the binding site for an essential viral protein, e.g. so-called TAR decoys (Sullenger et al, 1990).

Examples of genes which can be used in somatic gene therapy and which can be transferred into cells as components of gene constructs by means of the present invention include factor VIII (hemophilia A) (see, e.g. Wood et al., 1984), factor IX (hemophilia B) (see, e.g. Kurachi et al., 1982), adenosine deaminase (SCID) (see, e.g. Valerio et al., 1984), α-1 antitrypsin (emphysema of the lungs) (see, e.g. Ciliberto et al., 1985) or the cystic fibrosis transmembrane conductance regulator gene (see, e.g. Riordan, J.R. et al., 1989). These examples do not constitute a restriction of any kind.

As for the size of the nucleic acids, a wide range is possible: gene constructs of about 0.15 kb (in the case of a tRNA gene containing a ribozyme) to about 50 kb or more may be transferred into the cells by means of the present invention; smaller nucleic acid molecules may be applied as oligonucleotides.

It is clear that the widest possible applications are made possible precisely by the fact that the present invention is not subject to any limitations on the gene sequence and the fact that very large gene constructs may also be transferred by means of the invention.

Starting from the nucleic acid, the substance having an affinity for nucleic acid, preferably an organic polycationic substance, is determined, to ensure complexing of the nucleic acid, the obtained complexes preferably being substantially electroneutral. If the complexes contain, in addition to the endosomolytic conjugate, a conjugate of internalizing factor and substance having an affinity for nucleic acid, the cation component of both conjugates is taken into consideration with respect to the electroneutrality aspect.

In the course of earlier inventions it had been found that the optimum transfer of nucleic acid into the cell can be achieved if the ratio of conjugate to nucleic acid is selected so that the internalizing

factor-polycation/nucleic acid complexes are substantially electroneutral. It was found that the quantity of nucleic acid taken up into the cell is not reduced if some of the transferrin-polycation conjugate is replaced by non-covalently bound polycation; in certain cases there may even be a substantial increase in DNA uptake (Wagner et al., 1991a). It had been observed that the DNA of the complexes is present in a form compressed into toroidal structures with a diameter of 80 to 100 nm. The quantity of polycation is thus selected, with respect to the two parameters of electroneutrality and the achievement of a compact structure, while the quantity of polycation which results from the charging of the nucleic acid, with respect to achieving electroneutrality, generally also guarantees compacting of the DNA.

Thus, in a further embodiment of the invention, the complexes also contain nucleic acid-binding substances in a non-covalently bound form, which may be identical to or different from the binding factor, i.e. the substance with an affinity for nucleic acids in the conjugate. If the endosomolytic agent is free virus, the complexes comprise nucleic acid and internalizing factor conjugate. If an endosomolytic, e.g. a viral conjugate is employed, the nucleic acid is complexed with this conjugate, optionally in concert with a conjugate of an additional internalizing factor. The choice of non-covalently bound "free" substances having an affinity for nucleic acid, in their nature and quantity, is also determined by the conjugate(s), particularly taking account of the binding factor contained in the conjugate: if, for example, the binding factor is a substance which has no or limited capacity for DNA condensation, it is generally advisable, with a view to achieving efficient internalization of the complexes, to use substances having an affinity for DNA which possess this property in a high degree. If the

binding factor itself is a nucleic acid condensing substance and if it has already brought about compacting of the nucleic acid sufficient for effective internalization, it is advisable to use a substance having an affinity for nucleic acid which brings about an increase in expression by virtue of other mechanisms.

The suitable non-covalently bound substances having an affinity for nucleic acid according to the invention include compounds capable of condensing nucleic acid and/or of protecting it from undesirable degradation in the cells, particularly the substances of a polycationic nature mentioned hereinbefore. Another group of suitable substances comprises those which, by binding to the nucleic acid, bring about an improvement in the transcription/expression thereof, by improving the accessibility of the nucleic acid for the expression machinery of the cell. An example of a substance of this kind is chromosomal non-histone protein HMG1, which has been found to possess the capacity to compact DNA and promotes expression in the cell.

When determining the molar ratios of endosomolytic agent and/or internalizing factor/substance having an affinity for nucleic acid/nucleic acid(s), care should be taken that complexing of the nucleic acid(s) takes place, that the complex formed can be bound to the cell and internalized, and that, either by itself or with the aid of the endosomolytic agent, it is released from the endosomes.

The internalizing factor/binding factor/nucleic acid ratio depends particularly on the size of the polycation molecules and the number and distribution of the positively charged groups, criteria which are matched to the size and structure of the nucleic acid(s) to be transported. Preferably, the molar ratio of internalizing factor: substance having an affinity for a nucleic acid will range from about 10:1 to about 1:10.

After the construction and synthesis of the

conjugates and determination of the optimum ratio of conjugate:DNA for effective transfection, the quantity of the conjugate proportion which can be replaced, if desired, by free substance having an affinity for nucleic acid can be determined by titration. If polycations are used both as the binding factor and also as a free substance having an affinity for nucleic acid, the polycations may be identical or different.

For the embodiment of the invention which employs viral conjugates a method suitable for determining the ratio of the components contained in the complexes may consist in first defining the gene construct which is to be introduced into the cells and, as described above, finding a virus or virus component which is suitable for the particular transfection. Then the virus or virus component is bound to a polycation and complexed with the gene construct. Starting from a defined quantity of viral conjugate, titrations may be carried out by treating the target cells with this (constant) quantity of conjugate and decreasing concentrations of DNA, or vice versa. In this way the optimum ratio of DNA:virus conjugate is determined. If an additional internalizing factor is used the procedure may be, for example, to determine the optimum ratio of virus conjugate to internalizing factor conjugate starting from a constant quantity of DNA by titration.

The complexes may be prepared by mixing together the components i) nucleic acid, ii) viral conjugate, optionally iii) internalizing factor/binding factor conjugate, and optionally iv) non-covalently bound substance having an affinity to nucleic acid, all of which may be present in the form of dilute solutions. If polycations are used as a binding factor and at the same time as "free" polycations, it is generally advisable first of all to prepare a mixture of conjugates with "free" polycations and then combine this mixture with DNA. The optimum ratio of DNA to the

conjugate(s) and polycations is determined by titration experiments, i.e. in a series of transfection experiments using a constant amount of DNA and increasing amounts of conjugate(s)/polycation mixture. The optimum ratio of conjugate(s): polycations in the mixture is obtained by routine experimentation or by comparing the optimum proportions of the mixtures used in the titration experiments.

The DNA complexes may be prepared at physiological salt concentrations. Another possibility is to use high salt concentrations (about 2 M NaCl) and subsequent adjustment to physiological conditions by slow dilution or dialysis.

The most suitable sequence for mixing the components nucleic acid, conjugate(s), possibly free non-covalently bound substance with an affinity to nucleic acid is determined by prior experimentation. In some cases, it may prove advisable first to complex the nucleic acid with the conjugate(s) and then to add the free substance with an affinity for nucleic acid, e.g. the polycation, e.g. in the case of conjugates of transferrin-ethidium dimer and polylysine.

In a preferred embodiment of the invention, the internalizing factor or the additional internalizing factor, respectively, is transferrin and the binding factor is a polycation. The term "transferrin" denotes both the natural transferrins and also those transferrin modifications which are bound by the receptor and transported into the cell.

The nucleic acid is taken up in the form of complexes in which internalizing factor-polycation conjugates are complexed with nucleic acid. When there is a content of a non-covalently bound substance with an affinity for nucleic acid, this is preferably a polycation, which is either identical to or different from the polycation contained in the conjugate.

In the case of combination complexes the nucleic

acid is internalized in the form of complexes in which internalization factor conjugates on the one hand and endosomolytic conjugates on the other hand are complexed with nucleic acid.

The conjugates of internalizing factor and polycation, which are used together with free virus or together with the viral conjugates in the combination complexes, may be prepared by a chemical method or, if the polycation is a polypeptide, by a recombinant method; for methods of preparation, reference is made to the disclosure of EP 388 758.

The conjugates may also be prepared by connecting a glycoprotein, e.g. transferrin, and the binding factor to each other via one or more carbohydrate chains of the glycoprotein. Unlike the conjugates prepared by conventional coupling methods, conjugates of this kind are free from modifications originating from the linker substances used. In the case of glycoproteins which have only one or a few carbohydrate groups suitable for coupling, e.g. transferrin, these conjugates also have the advantage that they are precisely defined in terms of their binding site for glycoprotein/binding factor.

The quantity of endosomolytic agent used and the concentration thereof depend on the particular transfection being undertaken. It is desirable to use the minimum quantity of virus or virus component which is necessary to ensure the internalization of the virus and the nucleic acid complex and release from the endosomes. The quantity of virus (conjugate) is matched to the particular cell type and the infectivity of the virus for this type of cell must be taken into consideration above all. Another criterion is the particular conjugate of internalizing factor and binding factor, particularly with regard to the internalizing factor, for which the target cell has a specific number of receptors. Moreover, the quantity of virus (conjugate) will depend on the amount of DNA to be

imported. Generally, a small amount of virus is sufficient for a stable transfection which requires only a small amount of DNA, whereas a transient transfection, which requires larger amounts of DNA, requires a larger quantity of virus. For a particular application, preliminary tests are carried out with the target cells intended for transfection, possibly with a mixed cell population, and the vector system envisaged for the transfection, in order to determine the optimum virus concentration by titration, while the DNA used is conveniently a gene construct which largely coincides with the one intended for actual use, in terms of its size, and contains a reporter gene for easier measurement of efficiency of gene transfer. Within the scope of the present invention, the luciferase and ß-galactosidase genes have been shown to be suitable reporter genes for such tests.

Another aspect of the invention relates to a process for introducing complexes of nucleic acid, a nucleic acid binding substance and optionally an internalizing factor, into higher eucaryotic cells. The method is characterized in that the cells are brought into contact with an agent which has the ability of being internalized into the cells either per se or as a component of the nucleic acid complexes and of releasing the contents of the endosomes, in which the nucleic acid complexes are located after entering the cell, into the cytoplasm.

In general, it is preferred to apply nucleic acid complex and endosomolytic agent simultaneously, but they may also be applied one after the other. In case of separate applications, the sequence of application is not critical as long as the steps are carried out shortly after each other in order to guarantee that the components are in effective simultaneous contact. In the case of using free virus in a separate preparation, simultaneous administration of the preparation of virus

with the complexes may be guaranteed by having the virus preparation as part of the transfection medium which contains the nucleic acid complex. In the case of simultaneous administration of free virus, the nucleic acid complexes and virus preparation are mixed together before being administered.

In a preferred embodiment, the endosomolytic agent is a component of a combination complex.

In order to increase gene expression, the compositions according to the invention may also be administered repeatedly.

In a preferred embodiment, the cells are primary tumor cells. In a particularly preferred embodiment the nucleic acid is a DNA which contains one or more sequences coding for an immune modulating substance, preferably a cytokine.

In another embodiment the cells are myoblasts, preferably primary myoblasts.

In another embodiment the cells are fibroblasts, preferably primary fibroblasts.

In another embodiment the cells are hepatocytes, preferably primary hepatocytes.

In another embodiment the cells are primary endothelial cells.

In another embodiment the cells are primary airway epithelial cells.

In another embodiment the cells are T-cells.

In another embodiment the cells are B-cells.

Table 1 shows the transfection success of the present invention exemplified with various different cell types.

The composition of the invention was also investigated for transfection of canine hemophilia B fibroblasts. Luciferase and ß-galactosidase could be successfully expressed in these cells. Furthermore, the system was used to deliver the 1.4 kb canine factor IX cDNA into these fibroblasts. In a sandwich ELISA,

canine factor IX could be detected 24 hours after transfection.

In certain cases, it is advisable to use a lysosomatropic substance in addition to the endosomolytic agent, e.g. if the endosomolytic agent is a peptide conjugate or a retrovirus, the endosomolytic activities of which are not strictly pH-dependent.

It is known that lysosomatropic substances inhibit the activity of proteases and nucleases and may therefore inhibit the degradation of nucleic acids (Luthmann and Mangusson, 1983). These substances include chloroquine, monensin, nigericin and methylamine. Within the scope of the present invention it has been shown that monensin brings about an increase in the expression of reporter gene when a Moloney virus is used. The presence of chloroquine could be demonstrated to lead to expression of a reporter gene, imported by transferrin-mediated DNA transfer, in virtually 100% of K562 cells. BNL.CL2 or HepG2 hepatocytes did not respond as well to chloroquine as did K562 cells but they could be transfected to a level of 5 - 10% when exploiting the endosomolytic properties of added replication defective or chemically inactivated adenovirus.

With the aid of the present invention, the advantages of the biological vectors are increased. As a result of the distribution of the receptors there is a tropism both for internalizing factor and for the virus. By matching these two components to the particular cell population, it is possible to achieve a greater selectivity which is of particular importance in the therapeutic application of this invention. This aspect is of particular importance in therapeutic application of the present invention in the lungs, since the different cell populations in the lungs have different receptors, which may require the design of vectors with a higher binding affinity for a specific cell

population, e.g. for the ciliated cells of the respiratory tract. Ligands which may be used include lectins, for example. The design of such conjugates required inter alia the confirmation of the binding qualities of a possible ligand in the conjugate conformation. This confirmation can be carried out, for example, using antibodies against the ligand by means of immunohistochemical staining methods in the tissue where the composition is to be applied therapeutically.

In another aspect the present invention relates to pharmaceutical compositions containing as active ingredient a complex of therapeutically active nucleic acid, preferably as part of a gene construct, endosomolytic agent which is optionally conjugated and optionally an internalizing factor conjugate. Any inert pharmaceutically acceptable carrier may be used, such as saline, or phosphate-buffered saline, or any such carrier in which the DNA complexes have suitable solubility properties for use in the method of the present invention. Reference is made to Remington's Pharmaceutical Sciences, 1980, for methods of formulating pharmaceutical compositions.

The present invention offers the advantage of greatest possible flexibility for application, inter alia as pharmaceutical composition. The composition of the invention may occur as a lyophilisate or in a suitable buffer in deep-frozen state. It may also be provided as ready-to-use reagent in solution, preferably shipped and stored under refrigeration. Optionally, the components necessary for transfection, i.e. DNA, endosomolytic agent, optionally conjugated or ready for conjugation with a separate conjugation partner, DNA binding substance, optionally conjugated with an internalizing factor, optionally free polycation, may be present in a suitable buffer separate or partially separate as constituents of a transfection kit, which is also subject of the present invention. The transfection

kit according to the invention comprises a carrier which contains one or more containers such as test tubes, vials or the like which contain the equipment necessary for the transfection of the higher eucaryotic cells according to the present invention. In a transfection kit of this kind a first container may contain one or more different DNAs, e.g. coding for various antigens. A second container may contain one or more different internalising factor conjugates, enabling the transfection kit to be used as a modular system. Whether the constituents are supplied as a ready-to-use preparation or separately to be mixed immediately before use, depends, apart from the specific application, on the stability of the complexes, which can be determined routinely in stability tests. In a preferred embodiment, a transglutaminase-coupled adenovirus-polylysine conjugate, which has proven to be stable on storage, is used in one of the containers of a kit. In another preferred embodiment, biotinylated adenovirus and streptavidin-polylysine are kept in separate containers and mixed before application. One of ordinary skill in the art can design numerous different transfection kits to take advantage of the flexibility of the invention.

For therapeutic use, the composition may be administered systemically, preferably by intravenous route, as part of a pharmaceutical composition. The target organs for this application may be, for example, the liver, spleen, lungs, bone marrow and tumors.

One example for local application is the lung tissue (use of the composition according to the invention in fluid form for instillation or as an aerosol for inhalation). In addition to a high specificity of the ligand for the differentiated lung cells it may also be necessary, as a secondary measure, to influence various factors which are present in the environment of the lung tissue and which might interfere

with gene transfer (e.g. paralysis of the ciliary movement, breaking up of bronchial mucus, use of protease inhibitors). In addition, the pharmaceutical compositions of the invention may be administered by direct injection into the liver, the muscle tissue, into a tumor or by local administration in the gastro-intestinal tract. Another method of administration of the pharmaceutical composition is the application via the bile draining system. This method of application allows direct access to hepatocyte membranes at the bile canaliculi, avoiding interaction of the composition with blood constituents.

Recently, the feasibility of using myoblasts (immature muscle cells) to carry genes into the muscle fibres of mice was shown. Since the myoblasts were shown to secrete the gene product into the blood, this method may have a much wider application than treatment of genetic defects of muscle cells like the defect involved in muscular dystrophy. Thus, engineered myoblasts may be used to deliver gene products which either act in the blood or are transported by the blood. The experiments in the present invention have shown that both myoblast and myotube cultures, even primary ones, can be transfected with high efficiency. The most successful transfection media contained combination complexes of biotinylated adenovirus, transferrin-polylysine and streptavidin-polylysine. Besides the reporter gene products luciferase and ß-galactosidase, factor VIII was expressed in the muscle cells. Furthermore, the chicken adenovirus CELO was employed in combination complexes containing wheat germ agglutinin as an additional internalizing factor.

Therapeutic application may also be ex vivo, in which the treated cells, e.g. bone marrow cells, hepatocytes or myoblasts, are returned to the body e.g., Ponder et al., 1991, Dhawan et al. 1991. Another ex vivo application of the present invention concerns so-

called "cancer vaccines". The principle of this therapeutic approach is to isolate tumor cells from a patient and transfect the cells with a cytokine-encoding DNA. The next step may involve inactivation of the cells, e.g. by irradiation, in such a way that they no longer replicate but still express the cytokine. Then the genetically modified cells are applied to the patient from which they have been isolated, as a vaccine. In the environment of the vaccination site, the secreted cytokines activate the immune system, inter alia by activating cytotoxic T cells. These activated cells are able to exert their effect in other parts of the body and attack also non-treated tumor cells. Thus, the risk of tumor recurrency and of developing metastasis are reduced. A protocol suitable for the application of cancer vaccines for gene therapy was described by Rosenberg et al., 1992. Instead of retroviral vectors suggested by Rosenberg, the gene transfer system of the present invention may be used. In the experiments of the present invention primary melanoma cells were successfully transfected with a reporter gene contained in combination complexes of polylysine-coupled adenovirus and transferrin-polylysine.

The present invention can also be used in assays for determining the host immune response to a given antigen. Antigen-specific cytotoxic T lymphocytes (CTL) that kill infected cells play an important role in the host defence against viral infections or tumors. The interaction between T-cell and antigen-presenting cell (APC) is HLA (human lymphocytic antigens = MHC, major histocompatibility molecules)-restricted; to study CTL killing of cells expressing antigen in an <u>in vitro</u> CTL killing assay, one must present the antigen to the CTL in the correct HLA context, which usually means on an autologous target cell. A CTL-killing assay may be performed as follows: APCs are transfected with a DNA

construct containing an antigen encoding sequence. Antigen epitopes will be bound to MHC class I molecules and presented at the cell surface as a target for a specific CTL response. Thus, upon incubation with a sample of patient's serum, depending on the presence of specific CTLs, the APCs will be lysed. Lysis is measured by monitoring the release of e.g. radioactive chromium that was incorporated into the APCs prior to the addition of the serum. Established protocols (Walker et al., 1989) use B-LCLs (B-lymphoblastoid cell lines) induced to express antigen genes by transfection with recombinant vaccinia viruses. However, cells expressing antigen efficiently for about one day, die due to the lytic effect of vaccinia. These difficulties can be overcome by CTL killing assays employing the gene transfer system of the invention for introducing antigen encoding DNA constructs, e.g. constructs encoding HIV or tumor antigens into fibroblasts to render them antigen expressing. Primary fibroblasts are easy to obtain from biopsies, easy to grow, and have been demonstrated to be transfectable with a particularly high efficiency (about 50 to about 70 %) by means of the present invention. Such assays are useful for identifying epitopes recognized by Killer cells with a view to designing vaccines. Furthermore, they can be advantageously used in order to determine an individual's HLA restricted immune response against a given antigen.

Because a high level of expression of the transferred genes can be obtained in virtually all cells, the invention can be used to produce recombinant proteins. Here, there are no or few limitations as to the sequence and molecular weight of the transferred DNA, respectively. There is also a wide spectrum of cell types which are transfectable with the composition of the present invention. Thus, nearly any cell type can be used for the production of recombinant proteins which ensures that the recombinant protein is produced

in a faithful and fully modified post-translationally processed form guaranteeing high biological activity of the product.

Gene transfer into cells may be accomplished as shown in the Examples for luciferase and for FN-α, and practically any gene construct that gives rise to a desired protein product can be delivered. The desired protein product can be recovered from the transfected cell culture (either the cell supernatant or an appropriate cell homogenate, according to the protocol for the particular protein product), 24 hours to one week or more after the transfection.

The application of the gene transfer system according to the present invention for the production of recombinant proteins has the following advantages:

1) Due to the high transfection efficiency (more than 90% of the transfected cells can express the gene at high levels), no preselection of transfected cells is required and there is no need for establishing stable cell lines. Small scale cell culture can be sufficient to produce useful quantities of protein.

2) Large gene constructs may be delivered. Up to 48 kb have been successfully delivered thus far.

3) The gene expression can be performed in cells that guarantee the appropriate post-translational processing and modification (e.g. vitamin K-dependent carboxylation of clotting factors, see Armentano, et al., 1990, or cell type specific glycosylation).

4) A broader selection of target cell types is made available for gene expression using this method.

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In the Examples which follow, illustrating the present invention, the following materials and methods were used unless otherwise specified:

Preparation of transferrin-polylysine/DNA complexes

a) Human transferrin-polylysine conjugates

The method described by Wagner et al., 1991b, was used, in which polylysine is coupled to the carbohydrate side chains of transferrin.

A solution of 280 mg (3.5 μmol) of human transferrin (iron-free, Sigma) in 6 ml of 30 mM sodium acetate buffer, pH 5, was cooled to 0°C and 750 μl of 30mM sodium acetate buffer pH 5 containing 11 mg (51 μmol) of sodium periodate were added. The mixture was left to stand in the dark in an ice bath for 90 minutes. In order to remove the low molecular products, gel filtration was carried out, (Sephadex G-25, Pharmacia), yielding a solution which contained about 250 mg of oxidized transferrin (measured by ninhydrin assay). (In order to reveal the oxidized form which contains aldehydes and gives a color reaction when stained with anisaldehyde, the samples were added dropwise to a thin layer plate of silica gel and dried and the plates were dipped into p-anisaldehyde/sulfuric acid/ethanol (1/1/18), dried and heated.) The modified transferrin solution was added quickly (within 10 to 15 minutes) to a solution containing 1.5 μmol of fluorescein-labelled poly(L)lysine with an average chain length of 190 lysine monomers in 4.5 ml of 100 mM sodium acetate, pH 5. The pH of the solution was adjusted to pH 7.5 by the addition of 1 M sodium bicarbonate buffer. At intervals of 1 hour, 4 batches of 28.5 mg (450 μmol) of sodium cyanoborohydride were added to the mixture. After 17 hours, 2 ml of 5 M sodium chloride were added to adjust the solution to a total concentration of 0.75 M. The reaction mixture was loaded on a cation

exchange column (Pharmacia Mono S HR 10/10) and eluted with a salt gradient of 0.75 M to 2.5 M sodium chloride with a constant content of 25 mM HEPES, pH 7.3. The high salt concentration when loading the column and at the beginning of the gradient was essential for obtaining the polycation conjugates. Some transferrin (about 30%) together with a weak fluorescence activity was eluted in the flow through; the majority of fluorescence-labelled conjugate was eluted at a salt concentration of between 1.35 M and 1.9 M and was pooled in 3 fractions. These fractions (in the sequence in which they were eluted) yielded, after two lots of dialysis against 2 l 25 mM HEPES pH 7.3, a fraction A (TfpL190A) containing 45 mg (0.56 µmol) of transferrin, modified with 366 nmol of polylysine, a fraction B (TfpL190B) containing 72 mg (0.90 µmol) transferrin, modified with 557 nmol polylysine and a fraction C (TfpL190C), containing 7 mg (85 nmol) transferrin, modified with 225 nmol polylysine. If they were not used immediately, the transferrin conjugates were flash-frozen in liquid nitrogen and stored at -20°C in iron-free form. Before the incorporation of iron, samples (0.5 to 1 mg) were adjusted to a physiological salt concentration (150 mM) with sodium chloride. The iron was incorporated by adding 4 µl of 10 mM iron (III) citrate buffer (containing 200 mM citrate, adjusted to a pH of 7.8 by the addition of sodium bicarbonate) per mg of transferrin content. The conjugates containing iron were divided up into small aliquots before being used for DNA complexing, then flash frozen in liquid nitrogen or dry ice/ethanol and stored at -20°C. This procedure proved advisable once it was found that repeated thawing and freezing causes the conjugates to lose activity.)

b) Murine transferrin polylysine conjugates

A similar method was used as for human transferrin, in that coupling was effected by means of the

carbohydrate side chains. Conjugates of 15.5 nmol murine transferrin and 13 nmol pL290 were obtained from 4.1 mg (51 nmol) of murine transferrin and 2.1 mg (34 nmol) of pL 290.

Plasmid-DNA

a) pRSVL-DNA

6 µg of the DNA plasmid pRSVL (containing the Photinus pyralis luciferase gene under the control of the Rous Sarcoma Virus LTR Enhancer/Promoter (Uchida et al., 1977, De Wet et al., 1987), was prepared using the Triton-x Lysis standard method (Maniatis), followed by CsCl/EtBr equilibrium density gradient centrifugation, decolorizing with butanol-1 and dialysis against 10 mM Tris/HCl pH 7.5, 1 mM EDTA), in 350 µl HBS (150 mM NaCl, 20 mM HEPES, pH 7.3) was mixed with 12 µg of transferrin-polylysine conjugate in 150 µl HBS, 30 minutes before adding to the cells.

b) pCMV-DNA

The plasmid pCMV was prepared by removing the BamHI-Insert of the plasmid pSTCX556 (Severne et al., 1988), the plasmid was treated with Klenow fragment and the HindIII/SspI and Klenow-treated fragment from the plasmid pRSVL which contains the sequence coding for luciferase was inserted, or the sequence coding for β-galactosidase (Macgregor and Caskey, 1989) was used. Complexing was carried out analogously to pRSVL.

Production of Virus Preparations

a) Adenovirus preparations

The adenovirus strain d1312 described by Jones and Shenk, 1979, having a deletion in the E1a region was used. Replication of the virus was carried out in the E1a-trans-complementing cell line 293, and the

purification was carried out on a large scale as described by Davidson and Hassell, 1987. The purified virus was taken up in storage buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 0.1% BSA, 50% glycerol) or in HBS/40% glycerol and aliquots were stored at -70°C. The virion concentration was determined by UV-spectrophotometric analysis of the extracted genomic viral DNA (Formula: one optical density unit (OD, A_{260}) corresponds to 10^{12} viral particles/ml; (Chardonnet and Dales, 1970)).

b) Retrovirus-Preparation

The Moloney murine leukaemia retrovirus N2 was packaged in an ecotropic packaging line (Keller et al., 1985, Armentano et al., 1987). Supernatants from virus expressing cells were collected, flash frozen in liquid nitrogen and stored at -20°C. The supernatants used in the Examples had a titer of approximately 10^6 cfu/ml, as measured by neomycin-resistance colony formation with NIH3T3 cells. For the virus concentration experiments, the supernatants were passed through a 300 kD exclusion membrane (FILTRON) in an AMICON stirred cell concentrator under nitrogen pressure. Normally, 10 to 30 ml of supernatant were concentrated tenfold by this method.

Cells and Media

HeLa cells were cultivated in DMEM-Medium, supplemented with 5% heat-inactivated fetal calf serum (FCS), penicillin in amounts of 100 I.U./ml, streptomycin to 100 µg/ml and 2 mM glutamine. WI-38, MRC-5, and KB cells were cultivated in EMEM-medium (Eagle's modified essential medium), supplemented with 10% heat inactivated FCS, antibiotics as with DMEM medium, 10 mM non essential amino acids and 2 mM glutamine. CFT1, a respiratory cystic fibrosis

epithelial cell line (prepared by the method described by Yankaskas et al., 1991; the CFT1 cell line is characterized in that it is homozygous for the ΔF508 deletion CF-mutation) was cultivated in F12-7X-medium (Willumsen et al., 1989). For the gene transfer experiments the cells were cultivated in 6 cm cell culture plates until they were about 50% confluent (5 x 10^5 cells). The medium was removed and 1 ml of DMEM or EMEM/2% FCS medium was added. Then the conjugate-DNA complexes were added, followed immediately by the adenovirus d1312 (0.05 - 5.2 x 10^4 particles per cell) or a comparable volume of virus storage buffer (1 - 80 µl). The plates were returned to the incubator for one hour (5% CO_2, 37°C), then 3 ml of complete medium were added. After a further 24 hours' incubation the cells were harvested in order to measure the luciferase gene expression. In the case of the CFT1, the cells were cultivated for 4 hours in F12-7X medium without human transferrin before the gene transfer experiments.

The following cell lines were obtained from ATCC, obtainable under the Catalogue Numbers given; HeLa cells: CCL 2, K562 cells: CCL 243, HepG2 cells: HB 8065, TIB-73-cells: TIB 73 (BNL CL.2), NIH3T3 cells: CRL 1658, 293 cells: CRL 1573, KB cells: CCL 17, WI-38 cells: CCL 75, MRC 5 cells: CCL 171. H9 cells were obtained from the AIDS Research and Reference Reagent Program, U.S. Department of Health and Human Services, Catalogue Number 87.

Primary lymphocytes were obtained by taking up a 25 ml sample of umbilical cord blood in test tubes containing EDTA. Aliquots were underlayed with 4.5 ml of Ficoll-hypaque (Pharmacia) and centrifuged for 15 minutes at 2,500 rpm. The brownish layer between the upper plasma layer and the clear Ficoll layer was removed (about 10 ml). 40 ml of IMDM plus 10% FCS was added, the sample was centrifuged at 1200 rpm for 15 minutes and the cell pellet was suspended in 50 ml of

fresh IMDM plus 10% FCS (the cell density was about 2 x 10^6 cells/ml). A 250 µl aliquot of phytohaemagglutinin (PHA P, DIFCO) was added, the culture was incubated for 48 hours at 37°C and 5% CO_2, then recombinant IL-2 (BMB) was added (concentration: 20 units per ml). The cells were then split 1:3 with IMDM/20% FCS, 2 units/ml IL-2. Aliquots of the cells were deep frozen in liquid nitrogen in FCS plus 5% DMSO. Before use, the cells were grown in IMDM plus 20% FCS plus 2 units ml/IL-2.

For the sequential binding investigations HeLa cells were equilibrated at 4°C in 1 ml DMEM, supplemented with 2% FCS. The conjugate-DNA complexes were added as in the other tests and the plates were incubated for 2 hours at 4°C. Then the plates were exhaustively washed with ice cold DMEM/2% FCS, then 2 ml of this medium were added. Adenovirus dl312 or virus buffer was then added, the cells were left to warm up slowly, before being placed in the incubator for a further 24 hours. After this incubation, the cells were harvested and investigated for luciferase gene expression.

Luciferase Assay

The preparation of cell extracts, standardization of the protein content and determination of the luciferase activity were carried out as described by Zenke et al., 1990, Cotten et al., 1990, and in EP 388 758.

Example 1

Determination of the effect of the adenovirus treatment on gene transfer by transferrin-polylysine conjugates

First of all, the effect of an increase in dosage of virus on the ability of a defined amount of

conjugate-DNA complex to achieve gene transfer was investigated. For the complex formation, 6 µg of the plasmid pRSVL were mixed with 12 µg of human transferrin-polylysine conjugate (hTfpL190B). The conjugate-DNA complex plus various amounts of the adenovirus d1312 (0.05 - 3.2 x 10^6 virus particles per cell) were added to the HeLa cells. The results of this analysis are shown in Fig.1. The luciferase activity is expressed in light units of 50 µg of total cell protein. According to this analysis, increasing amounts of added adenovirus resulted in corresponding increases in gene transfer. The figure shows the averages from 2 to 4 separate experiments; the bars indicate standard deviation.

Example 2

Conjugate-DNA Complex Dosage Effect

Logarithmic dilutions of conjugate-DNA complexes prepared as in Example 1, were added to HeLa cells either with or without the addition of a constant dosage of adenovirus d1312 (1 x 10^4 viral particles per cell). The luciferase activity was determined as in Example 1. The results are shown in Figure 2.

Example 3

Enhancement of the gene transfer effected by transferrin polylysine by means of adenovirus occurs through receptor-mediated endocytosis

a) Effect of adenovirus treatment on the transfer of the complexed DNA

The following components were used for transfection:

6 µg pRSVL-DNA without transferrin-polylysine

conjugate (DNA); 6 μg pRSVL-DNA plus 6 μg of non-conjugated polylysine 270 (DNA + pL); 6 μg of pRSVL-DNA plus 12 μg of transferrin-polylysine conjugates used in previous examples (DNA + hTfpL190B). These transfection materials were added to the HeLa cells with or without adenovirus d1312 (d1312) (1 x 10^4 viral particles per cell). The preparation of the cell extracts, standardization for total protein and determination of the luciferase activity were carried out as in the previous examples. The results of the tests are shown in Fig. 3A.

b) Effect of adenovirus treatment on the transfer of receptor-bound DNA

Conjugate-DNA complexes (DNA + hTfpL190B) or polylysine-DNA complexes (DNA + pL) were bound to HeLa without being internalized, by incubating at 4°C. Non-bound complex was removed before the addition of adenovirus d1312 (d1312) (1 x 10^4 viral particles per cell) or a comparable buffer volume. Subsequent incubation was carried out at 37°C in order to permit internalization of the bound DNA complexes and adenoviruses. The luciferase activity was determined as described (Fig.3B).

c) Effect of adenovirus treatment of gene transfer by transferrin-polylysine conjugates

Conjugate-DNA complexes containing 6 μg pRSVL-DNA plus 12 μg transferrin-polylysine (DNA + hTfpL190B) were added to the HeLa cells with 1 x 10^4 adenovirus particles (d1312) per cell or a comparable quantity of heat-inactivated adenovirus d1312 (d1312 h.i.). Heat inactivation was carried out by incubating for 30 minutes at 45°C (Defer et al., 1990).

Example 4

Effect of adenovirus treatment on gene transfer by transferrin-polylysine conjugates in selected cell lines

Conjugate-DNA complexes (6 μg pRSVL + 12 μg hTfpL190B) were added to cells of the cell lines CFT1, KB, HeLa, WI38 and MRC5 with or without adenovirus d1312 (1×10^6 virus particles per cell). The efficiency of gene transfer for the various cell lines was determined as in the previous examples by luciferase assay (Fig.4).

Example 5

Enhancement of luciferase gene expression functions at the level of gene transfer, not at the level of transactivation

A cell line designated K562 10/6 constitutively expressing luciferase was prepared by transfecting cells with a plasmid which contained an RSV-luciferase gene fragment (an ApaI/PvuI fragment of pRSVL (De Wet et al., 1987)), cloned into the ClaI site of the pUCμ Locus (Collis et al., 1990). This plasmid was complexed with a transferrin-polylysine conjugate and K562 cells were transfected with these complexes, using the method described by Cotten et al., 1990. Since the pUCμ Locus plasmid contains a neomycin resistance gene it was possible to select for luciferase-expressing clones on the basis of neomycin resistance. For the further experiments, clone K562 10/6 was selected.

Aliquots of the parental cell line K562 (in 200 μl RPMI 1640 plus 2% FCS; 500,000 cells per sample) were treated either with 12 μg TfpL plus 6 μg pRSVL or with 4 μg pL 90 plus 6 μg pRSVL, in 500 μl HBS in either case. The quantities of adenovirus d1312 specified (Fig.5) were allowed to act on the cells for 1.5 hours at 37°C,

after which 2 ml of RPMI and 10% FCS were added. Then incubation was continued at 37°C for a further 24 hours and the cells were then prepared for measurement for the luciferase activity. It was found that incubation with adenovirus results in a significant increase in the luciferase activity (Fig.5A). This applies both to the TfpL complexes (2000 light units as against 25,000 light units) and also to the pL 90 complexes (0 as against 1.9 x 10^6 light units). This shows that the K562 cell line has the capacity to internalize pRSVL polylysine complexes and that this internalization, measured by luciferase expression, is significantly increased by the presence of adenovirus.

Analogous tests were carried out with the K562 10/6 cells which constitutively express the RSVL luciferase gene, and similar amounts of adenovirus d1312 were used. Aliquots of 500,000 cells (in 200 µl RPMI plus 2% FCS) were incubated at 37°C for 1.5 hours with the quantities of adenovirus d1312 specified in Fig.5B. Then, as in the parental cell line RPMI plus 10% FCS was added, incubation was continued for a further 24 hours and the luciferase activity was determined. As shown in Fig.5B, the treatment of these cells with the adenovirus does not have a detectable effect on the luciferase activity; the control values are in the same range as the values for the virus treated samples.

Example 6

Transfection of liver cells with asialofetuin-polylysine conjugates (AFpL) or with Tetra-galactose peptide-pL conjugates ((gal) 4pL) in the presence of adenovirus

a) Preparation of the lactosylated peptide

3.5 mg (1.92 µmol) of the branched peptide Lys-(Nε-Lys)Lys-Gly-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Gly-Cys, prepared by the Fmoc method using an Applied Biosystems

431A Peptide Synthesizer, containing a dithiopyridine group for Cys, were treated with a solution of 7.85 mg of lactose in 40 µl of 10 mM aqueous sodium acetate pH 5 at 37°C. To the solution were added four aliquots of 0.6 mg (10 µmol) of sodium cyanoborohydride at intervals of about 10 hours. After a total of 64 hours at 37°C 0.5 ml of HEPES pH 7.3 and 15 mg of dithiothreitol (DTT) were added. Fractionation by gel filtration (Sephadex G-10, 12 x 130 mm Eluent: 20 mM NaCl) under argon yielded 3.6 ml of solution of lactosylated peptide in the free mercapto form (1.73 µmol corresponding to the Ellmann test: 84% yield). The samples of modified peptide showed a color reaction with anisaldehyde but no color reaction with ninhydrin; this accords with the assumption that all 4 N-terminal amino groups are lactosylated. The tetra-galactose peptide-polylysine conjugate is shown in Fig. 6.

b) Preparation of 3-dithiopyridinepropionate-modified polylysine

400 µl of a 15 mM ethanol solution of SPDP (6.0 µmol) were added, with intensive mixing, to a gel-filtered solution of 0.60 µmol poly-L-lysine with an average chain length of 290 lysine monomers (pL290, hydrobromide, Sigma) in 1.2 ml of 100 mM HEPES pH 7.9. 1 hour later, 500 µl of 1 M sodium acetate pH 5 were added after gel filtration (Sephadex G-25) with 100 mM sodium acetate, the solution contained 0.56 µmol pL290 with 5.77 µmol of dithiopyridine linker.

c) Conjugation of the Peptide with Polylysine

Conjugates were prepared by mixing 1.5 µmol of the lactosylated peptide prepared in a) in 3 ml of 20 mM NaCl with 0.146 µl of the modified pL290 obtained from b) in 620 µl of 100 mM sodium acetate buffer under an argon atmosphere. After the addition of 100 µl of 2 M HEPES pH 7.9, the reaction mixture was left to stand for

18 hours at ambient temperature. By the addition of NaCl, the salt concentration was adjusted to 0.66 M and the conjugates were isolated by cation exchange chromatography (Pharmacia Mono S column HR 5/5; gradient elution, Buffer A: 50 mM HEPES pH 7.3; Buffer B: Buffer A plus 3 M NaCl). The product fractions eluted at salt concentrations of about 1.2 M - 1.8 M and were pooled in two conjugate fractions: the conjugate fractions were named (gal)4pL1 and (gal)4pL2. Dialysis against 25 mM HEPES pH 7.3 resulted in the conjugate fractions (gal)4pL1, containing 24 nmol of modified pL290 and (gal)4pL2, containing 24.5 nmol of modified pL290.

d) Preparation of asialofetuin conjugates

The conjugates were prepared on the same principle as the transferrin conjugates; a similar method of preparing asialoorosomucoid-polylysine conjugates was described by Wu and Wu in 1988.

The coupling of asialofetuin to polylysine was carried out by bonding via disulfide bridges after modification with the bifunctional reagent SPDP (Pharmacia). A solution of 100 mg (2.2 µmol) of asialofetuin (Sigma) in 2 ml of 100 mM HEPES pH 7.9 was subjected to gel filtration on a Sephadex G-25 column. 330 µl of a 15 mM ethanolic solution of SPDP (5.0 µmol) were added to the resulting 4 ml solution with vigorous stirring. After 1 hour at ambient temperature, purification was carried out by another gel filtration (Sephadex G-25); this resulted in 5 ml of a solution of 1.4 µmol asialofetuin, modified with 2.5 µmol of dithiopyridine linker.

Conjugates were prepared by mixing 1.4 µmol of modified asialofetuin in 5 ml of 100 mM HEPES pH 7.9 with 0.33 µmol of modified pL190 (containing 1.07 µmol of mercaptopropionate groups; the same process was used as for the preparation of the transferrin conjugates) in

6.5 ml of 200 mM HEPES pH 7.6, under an Argon atmosphere. The reaction mixture was left to stand for 24 hours at ambient temperature. The conjugates were isolated from the reaction mixture by cation exchange chromatography (Pharmacia Mono S-column HR 10/10; gradient elution, Buffer A: 50 mM HEPES pH 7.9; Buffer B: Buffer A plus 3 M sodium chloride) and sodium chloride was added until a final concentration of 0.6 M was achieved before loading the column. The product fraction eluted at a salt concentration of about 1.5 M. Dialysis with HBS yielded conjugates containing 0.52 µmol of asialofetuin, modified with 0.24 µmol of pL190.

e) Transfection of HepG2 cells with pRSVL-DNA complexes

HepG2 cells were grown in DMEM medium plus 10% FCS 100 I.U./ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine in T25 flasks. Transfections were carried out at a density of 400,000 cells per flask. Before the transfection, the cells were washed with 4 ml of fresh medium containing 10% FCS. Immediately before the transfection, chloroquine (Sigma) was added so that the final concentration in the cell suspension (plus DNA solution) was 100 µM.

10 µg pRSVL-DNA in 330 µl HBS were mixed with the quantities of TfpL190B conjugate (TfpL), asialofetuin pL90 conjugate (AfpL), polylysine 290 (pL) or Tetra-galactosepeptide polylysine conjugate (gal)4pL specified in Fig.7 in 170 µl of HBS. In the competition experiments, 240 µg of asialofetuin ((gal)4pL + Af) or 30 µg lactosylated peptide ((gal)4pL + (gal)4) were added after 30 minutes. The mixture was added to the cells; the cells were incubated at 37°C for 4 hours, then the transfection medium was replaced by 4 ml of fresh DMEM medium plus 10% FCS. After 24 hours the cells were harvested for the luciferase assay. The values given in Fig.7 represent the total luciferase

activity of the transfected cells. As shown in the figure, pL and TfpL show slight luciferase activities; (gal)4pL shows values as high as AfpL; (gal)4 or Af compete for the asialoglycoprotein receptor and, as expected, lower the values.

f) Transfection of HepG2 cells with pCMVL-DNA complexes

HepG2 cells were grown in 6 cm plates to a cell density of 300,000 cells per plate, as described in e). Before transfection, the cells were washed with 1 ml of fresh medium containing 2% FCS.

6 µg of pCMVL-DNA in HBS were mixed with the quantities of TfpL10B conjugate (TfpL), asialofetuin-pL conjugate (AFpL), polylysine290 (pLys290), (gal)4pL1 or (gal)4pL2 specified in Fig.8, in 170 µl HBS. After 30 minutes, 1 ml of DMEM, containing 2% FCS and 50 µl adenovirus stock solution d1312C, were added to each DNA-conjugate complex. In the competition experiments, 30 µg of lactosylated peptide (gal)4pL ((gal)4pL1 + (gal)4 or (gal)4pL2 + (gal)4) were added, as specified. The mixture was added to the cells; the cells were incubated for 2 hours at 37°C, then 1.5 ml of medium, containing 10% FCS were added. Two hours later, the transfection medium was replaced by 4 ml of fresh DMEM medium plus 10% FCS. After 24 hours the cells were harvested for the luciferase assay; the values in Fig. 8, represent the total luciferase activity of the transfected cells. pLys290 exhibits an effect, (gal)4pL exhibits a stronger effect; an addition of (gal)4, which competes for the asialoglycoprotein receptor, reduces the values to the value obtained for polylysine.

g) Transfection of TIB73 cells with pCMVL-DNA complexes

Cells of the embryonic murine liver cell line ATCC TIB73 (BNL CL.2; Patek et al., 1978) were grown at 37°C

in a 5% CO_2 atmosphere in "high glucose" DMEM (0.4% glucose), supplemented with 10% heat-inactivated FCS containing 100 I.U./ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine in 6 cm plates.

The transfections were carried out at a cell density of 300,000 cells per plate. Before the transfection, the cells were washed with 1 ml of fresh medium plus 2% of FCS.

6 µg pCMVL-DNA in 300 µl HBS were mixed with the specified amounts of murine transferrin-polylysine290 conjugate (mTfpL), asialofetuin-pL conjugates (AFpL), polylysine290 (pLys290), (gal)4pL1 or (gal)4pL2 in 170 µl HBS. After 30 minutes, 1 ml of DMEM, containing 2% FCS and 50 µl of adenovirus stock solution d1312 were added to each DNA conjugate complex. The mixture was added to the cells, the cells were incubated for 2 hours at 37°C, then 1.5 ml of medium containing 10% FCS was added. Two hours later, the transfection medium was replaced by 4 ml of fresh medium. After 24 hours the cells were harvested for the luciferase assay; the values shown in Fig. 9A represent the total luciferase activity of the transfected cells.

As a comparison, transfection was carried out without adenovirus in the presence of chloroquine: the transfection was performed at a cell density of 300,000 cells per plate. Before the transfection, the cells were washed with 1 ml of fresh medium containing 2% FCS. Immediately before transfection, chloroquine (Sigma) was added so that the final concentration in the cell suspension (plus DNA-solution) was 100 µM.
6 µg of pCMVL-DNA in 330 µl HBS were mixed with the specified amounts of mTfpL, AFpL, pLys290, (gal)4pL1 or (gal)4pL2 in 170 µl of HBS. After 30 minutes the DNA complexes were added to the cells. The cells were incubated for 2 hours at 37°C, then 1.5 ml of medium containing 10% FCS and 100 µM chloroquine were added. Two hours later the transfection medium was replaced by

4 ml of fresh medium. After 24 hours the cells were harvested for the measurement of luciferase. The values obtained for the luciferase activity are shown in Fig.9B.

Example 7

Introduction of DNA in T cells

a) Preparation of antiCD7 Polylysine190 conjugates

A solution of 1.3 mg of antiCD7 of antibody (Immunotech) in 50 mM HEPES pH 7.9 was mixed with 49 µl 1 mM ethanolic solution of SPDP (Pharmacia). After 1 hour at ambient temperature the mixture was filtered over a Sephadex G-25 gel column (eluent 50 mM HEPES Buffer pH 7.9), thereby obtaining 1.19 mg (7.5 nmol) of antiCD7, modified with 33 nmol pyridyldithiopropionate groups. Poly(L)lysine190, fluorescent labelled using FITC, was modified analogously with SPDP and brought into the form modified with free mercapto groups by treating it with dithiothreitol and subsequent gel filtration. A solution of 11 nmol of polylysine190, modified with 35 nmol mercapto groups, in 0.2 ml of 30 mM sodium acetate buffer was mixed with modified antiCD7 (in 0.5 ml 300 mM HEPES pH 7.9) with the exclusion of oxygen, and left to stand overnight at ambient temperature. The reaction mixture was adjusted to a content of about 0.6 M by the addition of 5 M NaCl. Isolation of the conjugates was carried out by ion exchange chromatography (Mono S, Pharmacia, 50 mM HEPES pH 7.3, salt gradient 0.6 M to 3 M NaCl); after dialysis against 10 mM HEPES pH 7.3, corresponding conjugates were obtained consisting of 0.51 mg (3.2 nmol) of antiCD7-antibody, modified with 6.2 nmol polylysine190.

b) Preparation of gp120-Polylysine 190 conjugates

Coupling was carried out by methods known from the

literature by thioether-linking after modification with N-hydroxysuccinimide ester of 6-maleimidocaproic acid (EMCS, Sigma) (Fujiwara et al., 1981).

Thioether-linked gp120-Polylysine 190-conjugates:

A solution of 2 mg of recombinant gp120 in 0.45 ml of 100 mM HEPES pH 7.9 was mixed with 17 µl of a 10 mM solution of EMCS in dimethylformamide. After 1 hour at ambient temperature, filtration was carried out over a Sephadex G-25 gel column (eluent 100 mM HEPES-Buffer 7.9). The product solution (1.2 ml) was immediately reacted, with the exclusion of oxygen, with a solution of 9.3 nmol polylysine 190, fluorescence-labelled and modified with 30 nmol mercapto groups (in 90 µl 30 mM sodium acetate pH 5.0), and left to stand overnight at ambient temperature. The reaction mixture was adjusted to a content of about 0.6 M by the addition of 5 M NaCl. The conjugates were isolated by ion exchange chromatography (Mono S, Pharmacia 50mM HEPES pH 7.3, salt gradient 0.6 M to 3 M NaCl); after fractionation and dialysis against 25 mM HEPES pH 7.3, 3 conjugate fractions A, B and C were obtained, consisting of 0.40 mg of rgp120 modified with 1.9 nmol polylysine 190 (in the case of Fraction A), or 0.25 mg rgp120 modified with 2.5 nmol polylysine 190 (Fraction B), or 0.1 mg rgp120 modified with 1.6 nmol of polylysine 190 (Fraction C).

pCMVL-DNA (6 µg/sample) were complexed with the specified amounts of polylysine90 or the specified polylysine conjugates in 500 µl HBS. In the meantime, aliquots of H9 cells (10^6 cells in 5 ml of RPMI with 2% FCS) or primary human lymphocytes (3 x 10^6 cells in Iscove's modified Dulbecco's medium (IMDM) plus 2% FCS) were prepared. The polylysine-DNA complexes were added to each cell sample. 5 minutes later, the specified amount of adenovirus d1312 was added. The cells were then incubated for 1.5 hours at 37°C, then 15 ml of RPMI

(in the case of H9 cells) or IMDM (in the case of the primary lymphocytes) plus 20% FCS were added to each sample. The cells were incubated for 24 hours at 37°C, harvested and treated as in the other examples, to determine the luciferase activity. The results of the tests carried out are given in Fig.10A (H9 cells) and Fig.10B (primary lymphocytes): in H9 cells, the antiCD7 conjugate (Fig.10A, lanes 7 to 9) and the gp120 conjugate (lanes 10 to 12) showed the best results in terms of the gene trarsfer achieved with adenovirus, while the gp120 conjugate achieved a clear expression of the luciferase gene even in the absence of adenovirus. It is worth noting that, in the tests carried cut, only the gp120 conjugate had the ability to intrcduce DNA into primary lymphocytes, and then only in the presence of defective adenovirus (Fig.10B, lanes 7 and 8).

Example 8

Inactivation of Adenoviruses

a) UV Inactivation

An adenovirus d1312 preparation, prepared and stored as described in the introduction to the Examples, was placed in 2 cm diameter wells of a cell culture plate (300 µl per well) on ice at an 8 cm spacing from 2 UV lamps (Philips TUV15 (G15 T8) lamps). The virus was exposed to the UV radiation for the times specified in Figure 11A and aliquots of each preparation were investigated for their virus titer and to determine whether and to what extent they were capable of augmenting gene transfer with polylysine-transferrin conjugates into HeLa cells.

The cultivation of the cells and the transfection were carried out essentially as described above under "cells and media"; the components used for transfection are shown in Fig.11A. The complexes of pCMVL-DNA and 12

µg TfpL were prepared in 500 µl HBS and added to 3×10^5 HeLa cells (in 1 ml DMEM plus 2% FCS). About 5 minutes later, 54 µl of each virus preparation was added to each culture and the culture was incubated at 37°C for one and a half to two hours. Then a 5 ml aliquot of DMEM plus 10% FCS was added to each culture, incubation was continued at 37°C for 24 hours and the cultures were harvested and investigated for luciferase activity. The quantity of 54 µl of non-irradiated virus is not in the saturation range, i.e. the test is sensitive to a quantity of virus at least 3 times greater. The results obtained for the luciferase expression are shown in Fig.11B (shaded rectangles).

The virus titer of each preparation was determined using the E1a complementing cell line 293. Serial dilutions of the non-irradiated and irradiated virus samples were prepared in DMEM plus 2% FCS. Parallel to this, samples of 5×10^4 293 cells were prepared (in a 2 cm well) in 200 µl DMEM plus 2% FCS. A 5 µl aliquot of each dilution was placed in each well. In order to allow the virus to bind to the cells, incubation was carried out at 37°C for one and a half hours, then 2 ml of DMEM plus 10% FCS were placed in each well. 48 hours later the cultures were examined in order to determine the cytopathic effect. The virus dilution above which less than 50% of the cells in the culture show a significant cytopathic effect after 48 hours indicates the relative amount of infectious virus in each virus preparation. The results obtained are shown in Fig.11B (open rectangles). The results of the tests carried out in this Example, show the decrease of 4 logs in the virus titer resulting from UV radiation is associated with only a twentyfold reduction in the luciferase gene transfer. This demonstrates that mechanisms which are crucial to the infectivity of the virus can be destroyed without affecting the ability of the virus to augment gene transfer.

It was observed that at low doses of the virus, the increase in gene transfer caused by the virus fell slightly (Fig.11A, lanes 3 to 6) and that this effect was more significant at the high doses (lanes 7 to 10).

b) Inactivation of Adenoviruses with Formaldehyde

2 ml of adenovirus preparation were passed over a 10 ml G25 column (Pharmacia PD 10G, 25M), pre-equilibrated with 150 mM NaCl, 25 mM HEPES pH 7.9, 10% glycerol, and taken up in a volume of 2.5 ml. Aliquots of the gel-filtered virus preparation were incubated without (0), with 0.01%, 0.1% or 1% formaldehyde for 20 hours on ice. Then Tris pH 7.4 was added to give a concentration of 100 mM, then the samples were dialyzed first for 2 hours against 1 liter of 150 mM NaCl, 50 mM Tris pH 7.4 and 50% glycerol and then overnight against 2 x 1 liter 150 mM NaCl, 20 mM HEPES pH 7.9 and 50% glycerol.

Aliquots of the virus were then examined for their titer on 293 cells (CPE endpoint assay or plaque assay, Precious and Russel, 1985). Then the effect of the formaldehyde-treated viruses on gene transfer into HeLa cells (300,000) was determined as in the previous examples by measuring the luciferase activity. 90 µl of the virus preparation, resulted in a DNA transfer corresponding to more than 10^3 light units. Treatment of the virus with 0.01% or with 0.1% formaldehyde resulted in a slight reduction in gene transfer activity (approximately tenfold reduction at 0.1%). Although the treatment with 1% formaldehyde causes a striking loss of gene transfer activity, 90 µl of the virus was still able to produce a gene expression corresponding to 10^4 light units.

In the treatment with 0.1% formaldehyde, a reduction in the virus titer to 10^5 PFU (plaque forming units) was coupled with a decrease in the luciferase activity of only 10%. The results of the test are shown

in Fig.12A.

c) Inactivation of Adenoviruses with long-wave UV + 8-methoxy psoralen treatment

Aliquots of purified virus were adjusted to 0.33 µg/µl 8-methoxy psoralen (stock concentration 33 µg/µl 8-methoxy psoralen dissolved in DMSO) and exposed to a 365 nm UV light source (UVP model TL-33), on ice, at a distance of 4 cm from the lamp filter. Exposure to the UV light was for 15-30 minutes, as indicated in Figure 12B. The virus samples were then passed over a Sephadex G-25 column (Pharmacia, PD-10) equilibrated with HBS + 40% glycerol and stored at -70°C.

Viral preparations were tested for either their acitivity in augmenting pCMVL/hTfpL conjugate delivery into HeLa cells (as evidenced by the resulting light units of luciferase activity, right-hand axes Fig. 12B) or for the ability to replicate in 293 cells (viral titer, left-hand axes Fig. 12B).

Example 9

Transfection of NIH3T3 cells with Moloney virus

In this and the Examples which follow, illustrating the increase in the internalization of transferrin-polylysine-DNA complexes by means of retroviruses, the following materials and methods were used, unless otherwise specified:

Transferrin-polylysine190 conjugates and conjugate-DNA complexes were prepared analogously to the preceding Examples with the difference that the complex forming reaction was carried out in a volume of 500 µl mM NaCl, 20 mM HEPES pH 7.4.

NIH3T3 cells were grown in DMEM medium with the addition of 10% FCS, 100 I.U./ml penicillin, 100 µg/ml

streptomycin and 2 mM glutamine. For the transfections, 5 to 7 x 10^5 cells per T25 flask were plated out 18 to 24 hours before transfection. Immediately before transfection, the cells were placed in fresh medium and the various components used for transfection were added in the following order: Chloroquine (100 µM, where stated), polylysine-transferrin-DNA complex and retrovirus preparation. The cells were then incubated for 4 hours at 37°C, and the medium was changed and the cells were harvested 24 hours later. Extracts were prepared using three freeze/thaw cycles; aliquots of the extract, standardized for protein content, were examined for luciferase activity as stated in the preceding Examples.

Under the conditions specified, transfections of 10^6 NIH3T3 cells were carried out with TfpL-DNA complexes in the presence of 100 µM chloroquine or without chloroquine as shown in Fig.13. It was found that without chloroquine the values for the luciferase activity reached only a background level (lane 1), whereas in the presence of chloroquine a high expression of the pRSVL reporter gene was measured (lane 2). Increasing amounts of the Moloney leukaemia virus, which were added to the cells at the same time as the DNA complexes, were able to increase the luciferase gene expression still further. (The amounts given in Fig. 13 are ml.)

Example 10

Investigation into whether the gene transfer effect can be attributed to the retrovirus

The virus preparation used in Example 9 was a crude, unfractionated supernatant of retrovirus expressing cells. In order to obtain evidence that the

increase in the DNA transfer achieved with this virus preparation could actually be ascribed to the virus, the supernatant was subjected to the dialysis/concentration purification described above, the retrovirus supernatant (shown as RVS in the drawing) being concentrated by a factor 10. If the retrovirus is responsible for the increase, the activity retained by the membrane, apart from any inactivation of the extremely unstable retrovirus during the concentration step, should be approximately 10 times that of the original supernatant. As in the previous Example, 10^6 NIH3T3 cells were transfected under the conditions given in Fig.14. Fig. 14 shows that the gene transfer increasing effect is present in the membrane retentate (20 to 600 µl were used, lanes 3 to 6). It was also found that 200 and 600 µl of the ten fold concentrated preparation are about half as active as 2 or 6 ml of the original, unconcentrated retrovirus preparation (lanes 7 and 8). Parallel tests were carried out with human K562 cells having no receptor for the ecotropic murine retrovirus. As expected, there was no increase in gene expression.

Example 11

Interactions between transferrin and its receptor play a role in the gene transfer effect of Moloney virus

In order to rule out the possibility that the transfer of TfpL/pRSVL complexes into the cells can be ascribed to non-specific binding of polylysine to the retrovirus, and in order to clarify the entry mechanism further, the retrovirus was examined for its ability to transport plasmid DNA, complexed only with polylysine, into the cell. The quantity of polylysine used corresponds to the optimum amount determined earlier which brings about total condensation of the plasmid DNA and is similar to the quantity of the polylysine used

with the polylysine-transferrin conjugate (Wagner et al., 1991a). The tests, the results of which are shown in Fig.15, demonstrated that the reporter gene, in the absence of chloroquine, is not expressed either in the form of TfpL-pRSVL complexes or in the form of pL-pRSVL complexes (lanes 1 and 2). In the presence of the retrovirus, on the other hand, the reporter DNA applied as a TfpL complex was expressed, but not in the form of pL-DNA complex (see lanes 3 and 4 together with lanes 5 and 6). Moreover, the tests carried out showed that the presence of excess free transferrin resulted in the reduction in the DNA transfer facilitated by the retrovirus (lanes 7 and 8). These results support the proposition that interactions between transferrin and its receptor play an essential part in augmenting the DNA uptake effected by the retrovirus.

Example 12

Influence of pH on the gene transfer effect of retroviruses

The experiments carried out in this Example were performed in order to examine the influence of the pH on the ability of retroviruses to augment gene transfer. The transfection experiments were carried out as in the preceding Examples. In order to establish whether a lower pH value is essential for the gene transfer effect, the two well-characterised inhibitors of endosome pH reduction, monensin and ammonium chloride, were used. It was assumed that these two substances would affect gene transfer if the retrovirus requires the lower pH value of the endosome for the gene transfer effect. If, on the other hand, other mechanisms come into play for this effect, namely direct fusion on the cytoplasm surface, as in the entry mechanism for HIV, these substances should either not

have a negative effect and may even have an enhancing effect if they modify the route of the TfpL-DNA complexes. The experimental results shown in Fig. 16 tend to support the latter hypothesis. The effect of both substances on TfpL-DNA transfer was investigated and it was found that neither of the two substances can functionally replace chloroquine. However, a slight increase in luciferase gene expression was found at higher ammonium chloride concentrations (lanes 1 to 5). The retrovirus alone shows the slight augmentation in DNA transfer as observed in the previous Examples (lane 6). A sharp increase was observed when the retrovirus was used in the presence of 1 µM monensin (lane 7). A less powerful effect was observed at a higher monensin concentration (lane 8) and in the presence of ammonium chloride (lanes 9 and 10).

Example 13

Augmentation of the gene transfer achieved by transferrin conjugates by means of the N-terminal endosomolytic peptide of influenza hemagglutinin HA2

a) Synthesis of the peptide

The peptide of the sequence (SEQ ID NO:1) of the Gly-Leu-Phe-Glu-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-Trp-Glu-Gly-Met-Ile-Asp-Gly-Gly-Gly-Cys was synthesized using the Fmoc (fluorenylmethoxycarbonyl) method (Atherton et al., 1979), using an Applied Biosystems 431A peptide synthesizer. The side chain protecting groups were t-butyl for Cys, Glu and Asp and trityl for Asn. After the coupling reaction, a ninhydrin test was carried out which showed a coupling level of > 98% for each step. Beginning with amino acid 19, double couplings were carried out. The N-terminal Fmoc group was removed from part of the peptide resin with 20% piperidine in NMP (N-methylpyrrolidone). Then the Fmoc-

protected and unprotected fractions were washed with DCM (dichloromethane) and dried under high vacuum. The yields were 293.6 mg Fmoc-free peptide resin and 366.5 mg of Fmoc-protected peptide resin. 111.1 mg of the Fmoc-free peptide resin was subjected to trifluoroacetic acid cleaving for one and half hours using a mixture of 10 ml TFA, 0.75 g of phenol, 300 µl of EDT (ethandithiol), 250 µl of Et-S-Me (ethylmethylsulfide) and 500 µl of water. The peptide was filtered from the resin through a sintered glass filter. The resin was washed with DCM and added to the filtrate. The filtrate was concentrated down to about 2 ml and then added dropwise with stirring to 40 ml of ether. The peptide deposit was removed by centrifuging and the ether supernatant was discarded. The precipitate was washed three times with 40 ml of ether and dried in a high vacuum. The 58 mg of crude product obtained were dissolved in 3.5 ml of 20 mM NH_4HCO_3, containing 300 µl of 25% NH_3/l. The solution was gel-filtered using the same buffer on a pre-packaged Sephadex G-25 column (Pharmacia PD-10). All the material was placed on a Mono Q column (Pharmacia 100 x 14 mm) (gradient: 0-10 min 100% A, 10-100 min 0-100% B. A: 20 mM NH_4HCO_3 + 300 µl NH_3/l. B: A + 3 M NaCl. Measured at 280 nm, Trp-fluorescence detection at 354 nm. Flow rate 1 ml/min). The product is eluted with 1 M NaCl. The main fraction of the Mono Q column, was further purified by reverse phase HPLC using a BIORAD-Hi-Pore RP 304 column (250 x 10 ml) (gradient 50 to 100% Buffer B in 12.5 min, 12.5 to 25 min 100% B. A: 20 mM NH_4HCO_3 + 300 µl NH_3/l, B: A in 98% methanol. Flow rate: 3 ml/min. Measured at 237 nm). The product is eluted at 100% B. The product fractions were evaporated down in a Speedvac, re-dissolved in buffer A and finally lyophilized. The yield was 8.4 mg of the HPLC-purified product in the cysteine-protected form. This peptide was designated

P16. In order to obtain the peptide in the free mercapto form, the t-butyl-protected substance was treated for 30 minutes at ambient temperature with thioanisol/ethanedithiol/trifluoracetic acid/trifluoromethanesulfonic acid (2/1/40/3; trifluoromethanesulfonic acid was added in the proportion specified after the other components). The peptide was isolated by ether precipitation and subsequent gel filtration (Sephadex G-25) using the above mentioned buffer A under an argon atmosphere.

b) Coupling of the influenza peptide to polylysine

b1) Direct binding via SPDP (Succinimidylpyridyl-dithiopropionate)

19.8 mg of polylysine (pL) 300 hydrobromide (Sigma) were gel-filtered on a Sephadex G-25 column (Pharmacia PD-10) in sodium acetate pH 5 in order to eliminate the low molecular fractions. On the basis of the ninhydrin test, the pL concentration after gel filtration was 3.16 mg/ml. The pH of the solution was adjusted to 7-8 using 1 M NaOH. 0.64 µmol of SPDP (Pharmacia: 40 mM solution in absolute EtOH) were added to 2.5 ml of the pL solution (7.9 mg pL = 0.13 µmol). This corresponds to a molar ratio of SPDP:pL of 5:1. The mixture was left to react overnight and gel-filtered in 20 mM NH_4HCO_3 pH 8.2 on a G25 column. After reduction of one aliquot of the filtrate with DTT (dithiothreitol) the measurement of thiopyridone showed that the reaction was complete. 0.3 µmol of pL-SPDP (based on µmol of PDP) in 2.212 ml were left to react with 0.35 µmol of peptide in the thiol form. A white precipitate which appeared when the peptide and pL were mixed was dissolved by adjusting the solution to 2 M guanidinium hydrochloride, the reaction taking place overnight. Photometric measurement of thiopyridone in the reaction mixture again confirmed that the reaction was complete. The mixture was then dialyzed twice against 2 liters of 20

mM HEPES/0.5 M guanidinium hydrochloride. The resulting solution was added to a Mono S column (0.7 x 6 cm, Pharmacia) (gradient: 0 to 20 min 100% A, 20-140 min 0-100% B. A: 20 mM HEPES pH 7.3/0.5 M guanidinium hydrochloride, B: 20 mM HEPES pH 7.3/3 M guanidinium hydrochloride, 0.3 ml/min. Detection at 280 nm and fluorescence detection at 354 nm, excitation at 280 nm). The product fraction which was eluted with 1.5 M guanidinium hydrochloride was dialyzed against 2 x 2 liters of HBS. Subsequent determination of the pL concentration by the ninhydrin test showed a concentration of about 1.14 mg/ml. The quantity of peptide in the solution of the conjugate was calculated from its absorption at 280 nm; this gave a molar ratio of peptide:pL of 4:1.

b2) Binding via a polyethyleneglycol linker

14.6 mg of pL 300 hydrobromide (Sigma) were gel filtered as described in b1). According to the ninhydrin test, the pL concentration after gel filtration was 4.93 mg/ml. The pH of the solution was adjusted to 7 - 8 with 1 M NaOH. 4.33 µmol SPDP (Pharmacia; 30 mM solution in absolute EtOH) were added to 2.7 ml of pL solution (13.3 mg pL = 0.22 µmol). This corresponds to a molar ratio of SPDP:pL of 20:1. After one and a half hours the reaction mixture was gel filtered on a Sephadex G-25 column in 0.1 M sodium acetate 3 M guanidinium hydrochloride. After reduction of one aliquot of the filtrate with DTT, thiopyridone was determined, indicating that the product fraction contained 3.62 µmol of SPDP. The SPDP-modified pL was reduced by adding 79 mg of DTT to the solution. After 2 hours reduction the solution was again filtered on G-25 under the conditions specified. The thiol measurement using the Ellman test showed a thiol concentration of 3.15 µmol in 2.224 ml.

17.6 mg = 5 µmol POE (Polyoxyethylene-bis(6-

aminohexyl), Sigma) were dissolved in 500 µl of 20 mM $NaHCO_3$/3 M guanidinium hydrochloride, pH 7-8, and reacted with 13.8 mg of EMCS (ε-maleimidocaproic acid-N-hydroxysuccinimide ester) (Sigma) (= 44.7 µmol), dissolved in 300 µl DMF (dimethylformamide). After 30 minutes, the solution was gel filtered on G-25 (20 mM $NaHCO_3$/3 M guanidinium hydrochloride). Photometric measurement of the maleimido group at 300 nm showed a concentration of 6.36 µmol of reacted EMCS in 2 ml of solution.

1.39 µmol of the peptide in thiol form (in 2.5 ml of 20 mM $NaHCO_3$/3 M guanidinium hydrochloride) were added dropwise to 1.049 ml of this solution (corresponding to 3.34 µmol EMCS) while the mixture was intensively mixed with a vortex in an argon current. After 15 minutes no more free thiol groups could be detected by the Ellman test.

The solution of the reduced SPDP-modified pL was adjusted to a pH of 7 - 8 by the addition of 1 M NaOH. 1.373 ml of this solution were added to the above reaction mixture while intensive mixing was carried out by means of a Vortex. This gave a molar ratio of peptide-SH:POE-EMCS:pL-SH of 1:2.4:1.4 (based on EMCS and SH). After 2.5 hours reaction, no more free thiol groups could be detected by the Ellman test. The material was dialyzed overnight against 2 liters of 20 mM HEPES pH 7.3/0.6 M NaCl and then added to a Mono S column (gradient 0 to 20 min 22% A, 20-150 min 22-100% B. A: 20 mM HEPES pH 7.3, B: A + 3 M NaCl. Flow rate 0.3 ml/min. UV-measurement was carried out at 280 nm and fluorescence measurement at 354 nm). The product which was eluted with 1.5 to 1.6 M NaCl was dialyzed against 2 liters of HBS. The measurement of the pL concentration using the ninhydrin test and photometric determination of the peptide concentration at 280 nm yielded a calculated pL ratio of 12:1 at a pL concentration of 0.49 mg/ml in a total volume of

4.5 ml.

c) Liposome preparation

Using the REV method (reverse phase evaporation) liposomes were prepared (Szoka and Papahadjopoulos, 1978; Straubinger and Papahadjopoulos 1983): aqueous phase 10 mM HEPES pH 7.3; 100 mM calcein; 150 mM NaCl; organic phase: a solution of 300 µmol L-α-lecithin (from egg yolk, chiefly palmitoyloleoylphosphatidylcholine; Avanti Polar Lipids) in 260 µl of chloroform was evaporated down using a rotary evaporator. The material was then dried in a high vacuum and then dissolved again in 3 ml of diethylether. 1 ml of the aqueous phase was thoroughly washed with the ether phase using a vortex and treated with ultrasound for 5 minutes at 0°C in a sonicator (bath type). After 30 minutes on ice, the material was treated with ultrasound for a further 10 minutes. The resulting stable emulsion was slowly evaporated down in a rotary evaporator. After the diethylether had been eliminated at 100 mbar, 0.75 ml of the aqueous phase were added. Residual traces of ether were eliminated by further evaporation at 50 mbar for 30 minutes. The resulting emulsion (1.7 ml) was centrifuged at 500 rpm and then extruded through a nucleopore polycarbonate membrane (0.1 µm), giving a final volume of 0.7 ml liposome solution. The liposomes were separated from the non-incorporated material by gel filtration (Sephadex G-50 medium, Pharmacia; 23 ml gel volume, 10 mM HEPES pH 7.3/150 mM NaCl). Six fractions of 500 µl were collected. Lipid phosphorus was determined using the method of Bartlett, 1959, at 2 mM.

d) Liposome Leakage Assay

The release of the liposome content (leakage) was measured by means of the emergence of the enclosed calcein and the resulting dilution which stops the quenching of fluorescence (Bondeson et al., 1984). The

calcein fluorescence was measured with a Kontron SMF 25 spectralfluorometer (excitation at 490 nm, emission at 515 nm). For this purpose, 100 µl aliquots of the above liposome solution were diluted 100 times with 0.1 M sodium acetate or 10 mM HEPES/150 mM NaCl buffer with the corresponding pH (4.3, 4.5, 5.0, 6.0, 7.3) in order to obtain a value of 1 ml. To these solutions were added 2.5 µg of the peptide (t-butyl-protected form; 1 µg/µl solution in HBS) in cuvettes, while mixing with a gentle stream of argon (final concentration 400 nM peptide). The calcein fluorescence was measured at different times after the addition of the peptide. The values for 100% leakage were determined by the addition of 2 µl Triton X-100 (Fluka).

The same procedure was used to measure the calcein fluorescence after the addition of peptide-pL conjugates to the liposome solution. 2.5 µg of the conjugate (1 µg/µl concentration based on the quantity of pL alone) were added to 1 ml of liposome solution (final concentration 20 nM modified peptide). Similarly, 2.5 µg of peptide-polylysine conjugate were subjected to the leakage assay after incubation with 5 µg DNA (15 minutes).

It was found that the peptide only causes the release of the liposome content in the acidic range (Fig.17). The peptide conjugate was active at a substantially lower pH, while even at a neutral pH a strong activity was found which was further increased as the pH was lowered. Complexing of the conjugate with DNA eliminated the activity at a neutral pH, whereas at an acidic pH there was a significant activity.

e) Transfection of K562-cells

K562-cells were grown in suspension in RPMI 1640 medium (Gibco BRL plus 2 g sodium bicarbonate/l) plus 10% FCS, 100 units per ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine up to a density of

500,000 cells/ml. 12 to 20 hours before transfection the cells were placed in fresh medium containing 50 µM desferrioxamine (this measure was taken to increase the number of transferrin receptors). On the day of transfection, the cells were collected, suspended in fresh medium containing 10% FCS plus 50 µM desferrioxamine (250,000 cells per ml) and 2 ml portions were placed in a dish with 24 wells.

6 µg of pCMVL-DNA in 160 µl HBS were mixed with the quantities of TfpL conjugate specified in Fig. 18 or with pL300 in 160 µl HBS, then after 15 minutes the specified amounts of influenza peptide-pL-conjugate (P16pL) were added and after a further 15 minutes the mixture was added to the K562 cells. The cells were incubated for 24 hours at 37°C and then harvested for the luciferase assay. The luciferase activity was determined as specified in the previous Examples. The values given in Fig. 18 represent the total luciferase activity of the transfected cells.

f) Transfection of HeLa cells

HeLa cells are cultivated in 6 cm culture dishes as described under "Cells and Media". The transfections were carried out at a density of 300,000 cells per plate. Before transfection, the cells were incubated with 1 ml of fresh medium containing 2% FCS.

6 µg of pCMVL-DNA in 160 µl HBS were mixed with the quantities of TfpL conjugate specified in Fig. 19 or with pL300 or a mixture of both in 160 µl HBS. After 15 minutes, the specified amounts of influenza peptide-pL-conjugates (P16pL) were added and after a further 15 minutes the mixture was added to the cells. The cells were incubated for 2 hours at 37°C, then 2.5 ml of fresh medium were added with an additional 10% FCS. The cells were incubated for 24 hours at 37°C and then harvested for the luciferase assay. The luciferase activity was determined as described in the preceding

Examples. The values given in Figure 19 represent the total luciferase activity of the transfected cells.

Example 14

Augmentation of the gene transfer achieved by transferrin conjugates by means of a second N-terminal endosomolytic peptide of influenza hemagglutinin HA2

a) Preparation of peptide-polylysine complexes

The peptide of the sequence (SEQ ID NO:2) Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-Trp-Glu-Gly-Met-Ile-Asp-Gly-Gly-Gly-Cys (designated P41) was synthesized in the same way as the peptide described in Example 13 a). The coupling of the peptide to polylysine (pL300) was performed as in Example 13 b1) by binding via SPDP. Thereby conjugates with a molar ratio of peptide:pL of 4:1 were obtained.

b) Transfection of HeLa cells with influenza peptide conjugates

HeLa cells were grown in 6 cm plates as specified and transfections were performed at a density of 300,000 cells per plate. Before the transfection, cells are incubated with 1.5 ml of fresh medium containing 2% FCS. 6µg pCMVL-DNA in 160 µl HBS (150 mM NaCl, 20 mM HEPES 7.3) were mixed with 6µg of TfpL190B conjugate in 160 µl HBS, after 15 min 10µg influenza peptide-polylysine-conjugate P41pL or, for comparison, 18µg of influenza peptide-polylysine-conjugate P16pL (see Example 13) were added (Fig. 20); the specified amounts of the two peptide conjugates had been tested to be optimal amounts for the augmentation of the gene transfer. After further 15 min the mixture was added to the cells. After 24 hours the cells were harvested for the luciferase assay. Values as shown in Fig. 20A represent the total luciferase activity of the transfected cells.

The comparison of the experiments with the two

peptide conjugates shows a more than 3.5 fold higher augmentation of the gene transfer obtained with the second peptide conjugate P41pL.

c) **Transfection of BNL CL.2 cells with influenza peptide conjugates**

BNL CL.2 cells were grown as described in Example 6. Influenza peptide P41 was conjugated with polylysine 300 at a molar ratio of peptide to polylysine of 1:1, 3:1 and 8:1. Complexes of 6 µg pCMVL DNA and 20 µg of the conjugates were added to the cells. For comparison, 20 µg of pL300 or 20 µg of P16 polylysine conjugate, prepared as described in Example 13, were used. The cells were incubated at 37°C for 4 h, then 2 ml of medium containing 18 % FCS was added. After 24 h, the cells were harvested for the luciferase assay, the results of which are shown in Fig. 20B. In the liposome leakage assay (Fig. 20C), which was performed as described in Example 13, the activity of the conjugates (at pH 5, equivalent to 2.5µg polylysine) increased with their content of peptide. (In the figure, P41 is designated "influ2")

Example 15

Transfection of HeLa cells with a ß-galactosidase reporter gene construct and <u>in situ</u> demonstration of ß-galactosidase expression

a) Culturing and transfection of cells

For the transfection, HeLa cells were grown in DMEM medium containing 5% FCS, penicillin, streptomycin and glutamine, as described in the previous Examples, in 3 cm culture dishes on cover slips (3×10^4 cells per dish).

For the transfection, 6 µg of the ß-galactosidase reporter gene construct (pCMV-ß-gal) in 160 µl of HBS

were complexed with 12 µg of TfpL190B in 160 µl of HBS and incubated for 30 minutes at ambient temperature.

In another experiment, 6 µg of pCMV-ß-gal in 160 µl of HBS were incubated with 6 µg of TfpL190B in 80 µl of HBS for 15 minutes at ambient temperature. Then 12 µg of the influenza peptide conjugate (P16pL) prepared in Example 13 in 80 µl of HBS were added and the mixture was incubated for a further 15 minutes. These DNA-polycation complexes were then mixed with 1 ml of DMEM plus 2% FCS, antibiotics and glutamine, as described above. In order to demonstrate the effect of chloroquine and adenovirus on the success of the transfection, in additional experiments chloroquine was also added to the medium containing the DNA polycation complexes, in a final concentration of 100 µM or 50 µl of the adenovirus strain solution d1312.

For the transfections, the original culture medium was removed from the cells and 1 ml of medium containing the DNA complexes with or without chloroquine or virus was added. After an incubation period of 2 hours at 37°C, 1 ml of DMEM containing 10% FCS, antibiotics and glutamine was added to the cells and incubation was continued for a further 2 hours. Then all the medium was removed and the cells were cultivated in 3 ml of fresh DMEM plus 10% FCS, antibiotics and glutamine.

b) ß-galactosidase assay

48 hours after transfection, the medium was removed, the cells were washed once with phosphate-buffered saline solution (PBS) and fixed with 0.5% glutardialdehyde in PBS for 5 minutes at ambient temperature. Then the fixative was discharged and the cells were washed once with PBS. Then incubation was carried out with the staining solution (10 mM phosphate buffer pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6 3H_2O$, 3.3 mM $K_3Fe(CN)_6$ and 0.2% 5-bromo-4-chloro-3-indolyl-ß-galactopyranoside) at 37°C for

20 minutes to 3 hours (Lim and Chae, 1989). Then the cover slips were rinsed in PBS, water and 96% ethanol, dried and mounted in Mowiol on slides. A Zeiss Axiophot Microscope was used for analysis.

Fig. 21 shows images of the microscopic magnifications (112 times). A: HeLa cells transfected with 6 µg pCMV-ß-gal, complexed with 12 µg TfpL190B. The staining reaction for ß-galactosidase was carried out for 3 hours. The Figure shows that very few cells (55 cells; a group of stained cells is indicated by an arrow) express the ß-galactosidase gene. B: HeLa cells transfected with 6 µg pCMV-ß-gal, complexed with 6 µg TfpL190B and 12 µg P16pL. Staining reaction: 3 hours. Few cells (250 cells) express the ß-galactosidase gene. However, the reaction of the cells is stronger than in A. C: HeLa cells transfected with 6 µg pCMV-ß-gal, complexed with 6 µg TfpL190B and 12 µg P16pL in the presence of 100 µM of chloroquine. Staining reaction: 3 hours. Numerous groups of cells show a strongly positive reaction (more than 1,000 cells). D: HeLa cells transfected with 6 µg pCMV-ß-gal, complexed with 12 µg TfpL190B in the presence of adenovirus d1312. Staining reaction: 20 min. Nearly all the cells (more than 90%) show a positive reaction. E: Non-transfected HeLa cells (control for the specificity of the ß-galactosidase reaction). Staining reaction: 3 hours.

Example 16

Transfection of HeLa cells with a 48 kb cosmid in the Presence of Adenovirus

a) Preparation of a cosmid containing the luciferase coding sequence

A 3.0 kb SalI fragment, containing a single P. pyralis luciferase coding sequence under control of the RSV promoter, was isolated from the plasmid p220RSVLuca

and ligated into the unique SalI site of the cosmid clone C1-7a1 to form concatamers. (C1-7a1 comprises a 37 kb human genomic DNA Sau3A fragment (partial digest), encoding no apparent genes, cloned into the BamHI site of the cosmid vector pW15 (Stratagene)). The ligation reaction product was then packaged in vitro and an aliquot of the resulting phage particles infected into E. coli MN544 and plated onto LB amp plates. The recombinants were screened by colony hybridization, using the 3.0 kb SalI fragment (^{32}P labelled by random priming) as a hybridization probe, and a number of positives analyzed by restriction mapping. A cosmid construct (CosLuc) containing a single copy of the SalI insert was grown and purified on a caesuim gradient (total size = 48 kb).

A small control cosmid pWELuc (12 kb) was prepared by digesting CosLuc with NotI, religating, transforming bacteria and isolating the appropriate plasmid. This resulted in a 12 kb DNA molecule lacking the human DNA insert and part of the polylinker of CosLuc. The plasmid pSPNeoLuc (8kb) is the plasmid described in Example 5 which contains an RSV-luciferase gene fragment (an ApaI/PvuI fragment of pRSVL, cloned into the ClaI site of the pUCμ Locus).

b) Delivery of the cosmid into HeLa cells

HeLa cells (3 x 10^4 cells per 6 cm dish) covered with 1 ml DMEM + 2% FCS were incubated with TfpL/DNA complexes prepared as described in the Introduction to the Examples, containing the indicated quantities of hTfpL, free polylysine and DNA. Incubation mixtures included, in addition, either 100 μM chloroquine (lanes 1 and 2) or 10 μl adenovirus d1312 containing 5 x 10^{11} particles per ml (lanes 3-12). After a 2 hour incubation at 37°C, 4 ml of DMEM + 10% FCS was added to each dish; 24 hours later, cells were harvested and luciferase activity was measured. Results are shown in

Fig. 22A.

c) Delivery of the cosmid into Neuroblastoma cells

Cells of a neuroblastoma cell line designated GI-ME-N (Donti et al., 1988) (1 x 10^6 cells per 6 cm dish) covered with 1 ml DMEM + 2% FCS were incubated with TfpL/DNA complexes prepared as described herein, containing the indicated quantities of hTfpL, free polylysine and DNA. Cell incubation mixtures included, in addition, either 100 µM chloroquine (lanes 3 and 4) or 10 µl adenovirus d1312 containing 5 x 10^{11} particles per ml, (lanes 5 and 6). After a 2 hour incubation at 37°C, 4 ml of DMEM + 10% FCS was added to each dish; 24 hours later, cells were harvested and luciferase activity was measured. Results are shown in Fig. 22B.

Example 17

Gene transfer by means of chemically coupled adenovirus-polylysine conjugates

a) Preparation of adenovirus-polylysine conjugates by chemical coupling

2.35 ml of a gel filtered (Sephadex G-25 PD10, Pharmacia) solution of adenovirus d1312 (approx. 10^{11} particles) in 150 mM NaCl/25 mM HEPES, pH 7.9/10% glycerol was mixed with 10 µl (10 nmol) of a 1 mM solution of SPDP (Pharmacia). After 3.5 hours at ambient temperature the modified virus was separated from the excess reagent by gel filtration (as above). The solution (2.5 ml) was purged with argon and allowed to react, under the exclusion of oxygen, under argon, with 42 µl of a solution of FITC-labelled polylysine (1 nmol), modified with 2.3 nmol of mercaptopropionate groups (prepared as described in EP 388 758). After 18 hours at ambient temperature half the solution was transferred into a centrifuge test-tube, carefully

covered with 1 ml of a cesium chloride solution (density 1.33 g/ml) and centrifuged at ambient temperature for 2 hours at 35000 rpm (SW60 rotor). The virus band was collected as 200 µl cesium chloride fraction and diluted to 1 ml with HBS/50% glycerol. A DNA binding assay was carried out with 300 µl of the modified virus: the virus solution was diluted with 1 ml HBS and mixed with 100 µl of solution of a ^{35}S-labelled DNA (15 ng pRSVL, prepared by Nick translation). As a control, the experiment was carried out in parallel with the same amount of unmodified virus dl312. After 30 minutes the samples were transferred into centrifuge tubes, carefully covered with 1 ml of a cesium chloride solution (density 1.33 g/ml) and centrifuged for 2 hours at 35000 rpm (SW60 rotor) at ambient temperature. The gradient was divided into 5 fractions; fraction 1, 1 ml; fraction 2, 0.6 ml, fractions 3-5, 200 µl each. The radioactivity of 200 µl portions of the fractions was measured and is shown in Fig. 23. The fractions which contain virus (3-5), especially fraction 3, show a significantly higher radioactivity than the control. This can be attributed to specific association of the polylysine-modified adenovirus with the labelled DNA.

b) Transfection of K562 cells

K562-cells (ATCC CCL 243) were grown in suspension in RPMI 1640 medium (Gibco BRL, plus 2 g sodium bicarbonate/l) plus 10% FCS, 100 units per ml penicillin, 100 µl/µl streptomycin and 2 mM glutamine) up to a density of 500,000 cells/ml. 12 to 20 hours before transfection the cells were placed in fresh medium containing 50 µM desferrioxamine (this measure was taken to increase the number of transferrin receptors). On the day of transfection, the cells were collected, suspended in fresh medium containing 10% FCS plus 50 µM desferrioxamine (250,000 cells per ml) and 2 ml portions were placed in a dish with 24 wells.

The specified amounts of pCMVL-DNA (6, 0.6, 0.06 µg) in 100 µl of HBS were mixed with 50 µl of polylysine adenovirus (pLadeno) or corresponding amounts (35 µl) of control adenovirus dl312. After 20 minutes, corresponding amounts (12, 1.2, 0.12 µg) of TfpL190B conjugate in 150 µl of HBS were added. After a further 20 minutes the mixture was added to the K562 cells. The cells were incubated for 24 hours at 37°C and then harvested for the luciferase assay. The luciferase activity was determined as in the preceding Examples. The values given in Fig. 24 represent the total luciferase activity of the transfected cells.

c) Transfection of HeLa cells

One method of testing the activity of a polylysine-virus conjugate is by checking the conjugate for its ability to transport very small amounts of DNA (less than 0.1 µg). An increased DNA transfer capacity was expected when the adenovirus is directly bound to the polylysine-condensed DNA, as the internalizing factors (transferrin and adenovirus fiber protein) are directly associated with the DNA which is to be transported. To test this assumption, a constant quantity of the polylysine-adenovirus conjugate (2.5 µl, about 5×10^7 virus particles) was complexed with different amounts (3 µg to 0.0003 µg) of reporter plasmid in 475 µl of HBS. After 15 minutes incubation at ambient temperature a quantity of transferrin-polylysine corresponding to the mass of DNA was added to each sample (this quantity of TfpL was selected because it guarantees total "packaging" (electroneutrality) of 50% of the plasmid DNA and at the same time ensures binding space for the virus-polylysine conjugate. After the addition of TfpL the mixtures were incubated for 15 minutes, then each mixture was placed in a 6 cm culture dish containing 300,000 HeLa cells in 1 ml of DMEM/2% FCS. Then the cells were incubated for 1.5 hours at 37°C, then 4 ml of

DMEM/10% FCS were added. In parallel, equivalent quantities of DNA were complexed with a two-fold mass excess of TfpL (the quantity for total DNA condensation) and used for the gene transfer into HeLa cells (once on its own and once in the presence of 25 µl of the non-polylysine-coupled adenovirus d1312 preparation). After 24 hours the cells were harvested, extracts were prepared and aliquots were examined for luciferase activity. The results of these tests are shown in Fig. 25: in the absence of adenovirus, no luciferase activity can be detected in a quantity of DNA less than 0.3 µg. Both polylysine-coupled and non-coupled adenovirus functioned well with large quantities of DNA (3 µg and 0.3 µg). However, with the non-coupled adenovirus there was an approximately 100 fold fall in activity at 0.03 µg and negligible activity below this amount of DNA. By contrast the polylysine-coupled virus retains its gene transfer capacity both at 0.003 and at 0.0003 µg of DNA. This quantity of DNA corresponds to about 100 DNA molecules per cell and about 1 virus particle per DNA molecule.

Example 18

Gene transfer by means of adenoviruses enzymatically coupled to polylysine

a) Enzyme reaction

2 ml of the adenovirus preparation (strain d1312; 5×10^{10} PFU/ml) were applied to a Sephadex G-25 gel filtration column (Pharmacia) equilibrated with 25 ml of reaction buffer (0.1 M Tris-HCl; pH 8.0, 2 mM DTT, 30% glycerol). Elution was carried out with 3.5 ml of reaction buffer. The reaction mixture for enzymatic coupling consists of 1150 µl of the virus elution fraction, 0.5 nmol guinea-pig liver transglutaminase (TG) (Sigma), 2 nmol or 20 nmol of Polylysine290, 10 mM

$CaCl_2$ and reaction buffer in a final volume of 1500 µl. The reaction was carried out at 37°C for 1 hour and then stopped by the addition of 30 µl of 0.5M EDTA. In order to monitor the specificity of the coupling, reaction mixtures were also prepared without transglutaminase. Non-incorporated polylysine was separated from the viruses by centrifuging in a CsCl-gradient (density 1.33 g/ml; 170,000 x g, 2 hours). The fraction containing the viruses was collected, mixed with an equal volume of glycerol, frozen in liquid nitrogen and stored at -70°C.

b) Demonstrating the binding of polylysine to adenoviruses

The reaction was carried out as described above with polylysine which had been labelled with ^{125}I with Bolton-Hunter reagent (Amersham). After the CsCl-gradient centrifugation the virus fraction was drawn off and separated by means of another CsCl gradient. The gradient was then fractionated and the radioactivity in every fraction was determined using a scintillation counter. As shown in Fig. 26, it became apparent that in the reaction mixture with TG (d1312/TG-pL), radioactive polylysine had accumulated in the virus fraction (virus). In the control mixture without TG (d1312/pL) there was no accumulation of radioactive polylysine in the virus fraction.

c) Testing the polylysine-modified adenovirus fractions for their effect on the efficiency of transfection

i) Cells and media

For the transfection, $5x10^5$ cells (murine hepatocytes; ATCC No.: TIB 73) in DMEM with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 I.U./ml penicillin and 100 µg/ml of streptomycin were seeded in 6 cm culture dishes.

ii) Formation of the virus-DNA-transferrin complexes

50 µl of the polylysine-modified virus fraction were mixed with 6 µg of the DNA plasmid pCMVL in 10 µl HBS and incubated for 20 minutes at ambient temperature. Then 8 µg of murine transferrin-polylysine290B (mTfpL) were added to the mixture and incubation was continued for a further 10 minutes.

iii) Transfection of the murine hepatocytes

The virus-DNA-transferrin complexes were mixed with 1.5 ml of medium (DMEM with 2% FCS, 2 mM glutamine and antibiotics) and added to the cells, after removal of the old medium. After 2 hours incubation at 37°C, 2 ml of DMEM with 10% FCS, glutamine and antibiotics were added to the cells. After a further 2 hours cultivation the entire medium was removed and 4 ml of fresh DMEM with 10% FCS, glutamine and antibiotics were added to the cells.

iv) Determining the luciferase expression

24 hours after transfection the cells were harvested and the luciferase assay was carried out as described above.

As can be seen from Fig. 27, the virus preparation in which the adenoviruses had been treated with TG and 20 nmol of polylysine (d1312/TG-20 nmol pL) showed the strongest expression (153540000 light units). The virus preparation with TG and 2 nmol of polylysine (d1312/TG-2 nmol pL) was somewhat less active (57880000 light units). The control fraction in which the adenoviruses were treated with 20 nmol of polylysine but with no TG was less effective by a factor of 500 approximately. As a comparison, further complexes were used for transfection with the initial preparation of adenoviruses treated neither with TG nor with polylysine (d1312). This preparation yielded 4403000 light units.

d) Increasing the transfection efficiency by polylysine-modified adenoviruses compared with unmodified adenoviruses, particularly with small amounts of DNA

Transfection was carried out as described in Example 3c), using 50 µl of the adenovirus fraction d1312/TG-20 nmol pL and 6 µg pCMV-Luc/8 µg mTfpL, 0.6 µg pCMVL(=pCMV-Luc)/0.8 µg mTfpL or 0.06 µg pCMV-Luc/0.08 µg mTfpL for complexing. As a comparison, transfections were also carried out with 6 µg, 0.6 µg, 0.06 µg pCMV-Luc/mTfpL complexes and unmodified adenoviruses (d1312). It was found that the complexes with polylysine-modified adenoviruses yielded high expression levels even with small amounts of DNA, whereas expression was sharply reduced with unmodified adenoviruses (Fig. 28).

Example 19

Gene transfer with conjugates in which the binding between the adenovirus and polylysine is obtained by means of a biotin-streptavidin bridge

a) Biotinylation of adenovirus d1312

2.4 ml of a gel filtered (Sephadex G-25 PD10, Pharmacia) solution of adenovirus d1312 (about 10^{11} particles) in 150 mM NaCl/5 mM HEPES, pH 7.9/10% glycerol, was mixed with 10 µl (10 nmol) of a 1 mM solution of NHS-LC biotin (Pierce 21335). After 3 hours at ambient temperature the biotin-modified virus was separated from the excess reagent by gel filtration (as above). The solution was adjusted to a glycerol concentration of 40% by adding glycerol (total volume 3.2 ml) and stored at -25°C. The biotinylation of the virus was demonstrated by qualitative detection after

vacuum dryer, blocking with BSA, incubating with streptavidin-conjugated alkaline phosphatase (BRL), washing and incubating for 1 hour with the developing solution NBT/X-phosphate (nitro blue-tetrazolium salt/5-bromo-4-chloro-3-indolylphosphate, toluidine salt; Boehringer Mannheim) a positive color reaction was found.

b) Preparation of streptavidin-polylysine conjugates

The coupling of streptavidin to polylysine was effected using the method described by Wagner et al., 1990, and in EP-A1 388 758.

79 nmol (4.7 mg) of streptavidin in 1 ml of 200 mM HEPES pH 7.9 and 300 mM NaCl were treated with a 15 mM ethanolic solution of SPDP (236 nmol). After 1.5 hours at ambient temperature the modified protein was gel filtered over a Sephadex G-25 column, thereby obtaining 75 nmol of streptavidin, modified with 196 nmol of dithiopyridine linker. The modified protein was reacted under an argon atmosphere with 3-mercaptopropionate-modified polylysine (75 nmol, average chain length 290 lysine monomers, modified with 190 nmol mercapto-propionate linker) in 2.6 ml of 100 mM HEPES pH 7.9, 150 mM NaCl. Conjugates were isolated by cation exchange chromatography on a Mono S HR5 column (Pharmacia). (Gradient: 20 - 100% buffer B. Buffer A: 50 mM HEPES pH 7.9; buffer B: buffer A plus 3 M sodium chloride). The product fraction eluted at a salt concentration of between 1.2 M and 1.7 M. Dialysis against HBS (20 mM HEPES pH 7.3, 150 mM NaCl) resulted in a conjugate consisting of 45 nmol of streptavidin and 53 nmol of polylysine.

c) Transfection of HeLa cells

HeLa cells were grown in 6 cm culture dishes as described in Example 1. The transfections were carried out at a density of 300,000 cells per plate. Before the

transfection the cells were incubated with 1 ml of fresh medium containing 2% FCS.

6 µg of pCMVL-DNA in 100 µl HBS were mixed with 0.8 µg of streptavidin-polylysine in 170 µl of HBS. After 20 minutes, 3 µg of polylysine pL300 in 170 µl of HBS were added. After another 20 minutes, 65 µl of biotinylated adenovirus or, as the control, corresponding amounts of adenovirus d1312 (30 µl, starting virus for modification), were added. The complex mixtures, ("biotinAdV/complex A" or "control AdV", see Fig. 29) were left to stand for a further 20 minutes.

Alternative complexing was carried out by mixing 65 µl of biotinylated adenovirus first with 0.8 µg of streptavidin-polylysine in 50 µl HBS then adding 6 µg of pCMVL-DNA in 170 µl of HBS after 20 minutes, and a further 20 minutes later adding 3 µl of polylysine pL300 in 200 µl HBS. (Complex mixture "biotinAdV/complex B").

0.6 µg of pCMVL-DNA in 67 µl HBS were mixed with 0.3 µg of streptavidin-polylysine in 33 µl of HBS. After 20 minutes, 65 µl of biotinylated adenovirus or, as the control, corresponding quantities of adenovirus d1312 (30 µl, starting virus for modification) were added. The complex mixtures ("biotinAdV/complex A" or "control AdV", see Fig. 29) were left to stand for a further 20 minutes and then diluted to 500 µl with HBS. Alternative complexing was carried out by mixing 65 µl of biotinylated adenovirus first with 0.3 µg of streptavidin-polylysine in 50 µl of HBS and after 20 minutes adding 0.6 µg of pCMVL-DNA in 50 µl HBS. The complex mixture ("biotinAdV/complex B") was left to stand for a further 20 minutes and then diluted with HBS to 500 µl.

The mixtures were added to the cells, the cells were incubated for 2 hours at 37°C, then 2.5 ml of fresh medium containing 10% added FCS were added. The cells were incubated for 24 hours at 37°C and then harvested

for the luciferase assay. The luciferase activity was determined as described in the preceding Examples. The values given in Fig. 29 represent the total luciferase activity of the transfected cells.

In parallel, transfections of HeLa cells were carried out using as the virus component of the conjugate a biotinylated virus which had been inactivated by psoralen/UV-treatment. Inactivation was carried out as follows: 200 µl batches of biotinylated virus preparation were placed in two wells of a 1.6 cm tissue culture plate. 2 µl (33 mg/ml) of 8-methoxypsoralen (in DMSO) were added to each sample, the dish was placed on ice and irradiated for 10 minutes with a UV lamp (365 nm; UVP TL-33 lamp) with the sample being 4 cm from the filter. After the irradiation the two samples were combined and gel filtered (G50, Nick column, Pharmacia), the column having previously been equilibrated with 40% glycerol in HBS. Aliquots of 75 µl were complexed with 0.8 µg of streptavidin-polylysine and used for the transfection of HeLa cells as described above.

By cytopathic end point assay it was established that the virus titer was reduced by a factor of more than 10^4 by the inactivation, whereas the transfer capacity was reduced by less than 50% at high concentrations and by a factor of 5 at low concentrations.

d) Transfection of K562 cells

K562 cells were grown in suspension in RPMI 1640 medium (Gibco BRL, plus 2 g sodium bicarbonate per 1 liter) plus 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine reaching a density of 500,000 cells/ml. At 16 hours before transfection, the cells were placed in fresh medium containing 50 µM desferrioxamine (Sigma). The morning of the transfection, the cells were collected,

resuspended in fresh medium containing 10% fetal calf serum plus 50 µM desferrioxamine at 250,000 cells per ml, and placed in a 24-well dish. 2 ml per well.

Three different types of DNA complexes were prepared: a) A solution of 6µg pCMVL-DNA in 160 µl HBS (150 mM NaCl, 20 mM HEPES 7.3) was mixed with 12µg of TfpL190B conjugate in 160 µl HBS, after 30 min 20 µl of adenovirus dl312 was added and the mixture was added to the cells. b) A solution of 800 ng streptavidin-polylysine in 160µl HBS was mixed with 20µl of biotinylated adenovirus, prepared as described in a), after 30 min a solution of 6µg pCMVL-DNA in 160 µl HBS was added, and after further 30 min the solution was mixed with 10µg TfpL190B conjugate in 160 µl HBS. After 30 min the mixture was added to the cells.
c) The DNA complexes were prepared analogously to b) with the difference that instead of TfpL190B conjugate a solution of 3.5µg poly(L)lysine p(Lys)290 was added. The cells were incubated at 37°C for 24 hours and then harvested for the luciferase assay. Values as shown in Fig. 30 represent the total luciferase activity of the transfected cells.

Example 20

Gene transfer into Primary Bone Marrow Cells

a) Isolation of Bone Marrow Cells

Primary bone marrow cells were harvested from mice by flushing culture medium (IMDM containing 10% FCS, $5x10^{-5}$M ß-mercaptoethanol, 1% IL-3 conditioned medium and antibiotics) with an injection needle (0.4mm or 0.5mm in diameter) attached to a 1 ml syringe through the isolated femura and tibiae. The cells were then washed once in culture medium by centrifugation at 100xg for 8 min. Thereafter the cells were resuspended at a concentration of 10^7 cells/ml and seeded into T25 culture

flasks. After 4h the non-attached cells were transferred into a new T25 culture flask and cultured overnight in the presence of 50µM desferrioxamine.

b) Formation of adenovirus-transferrin-polylysine-DNA complexes

For formation of complexes 50µl of biotinylated adenovirus were incubated with 400ng of streptavidin-modified polylysine in 20µl HBS for 20 min. Then 20µl of HBS containing 6µg pCMVL were added. After an incubation period of 20 min 7 µg of mouse transferrin-polylysine conjugate (mTfpL) in 160µl HBS were added and the whole mixture was incubated for further 20 min.

c) Transfection

For transfection the bone marrow cells were recovered from the culture medium by centrifugation at 100xg for 8 min. The cell pellet was resuspended in 3ml of culture medium containing 2% FCS and 250µl of the adenovirus-DNA-transferrin complexes, and cultured in a new T25 flask for 3h at 37°C. Then 3ml and after a period of 2h a further 6ml of culture medium containing 10% FCS were added.

d) Determination of luciferase expression

The cells were harvested 48h after transfection and analyzed for expression of luciferase as described in the other Examples. The transfection led to an expression of luciferase activity corresponding to $310x10^3$ light units/100µg total cell protein.

Example 21

Transfection of Neuroblastoma cells with a 48 kb cosmid in the presence of free adenovirus and adenovirus-polylysine conjugates

Cells of a cell line designated GI-ME-N were transfected, as described in Example 16, with the 48 kb cosmid, with the specified quantities of hTfpL, free pL and DNA. The incubation mixtures included, in addition, either 100 μM chloroquine (lanes 3 and 4) or 10 μl adenovirus dl312 containing 5 x 10^{11} particles per ml, (lanes 5 and 6). The last two samples (lanes 7 and 8, StpL/Biotin) contained 15 μl biotinylated adenovirus dl312 (1 x 10^{11} particles) incubated with streptavidin-polylysine (0.8 μg prepared as in Example 19) for 30 minutes in 150 μl HBS. 6 μg DNA in 150 μl HBS was then added to the sample for 30 minutes at room temperature, followed by 150 μl HBS containing 6 μg hTfpL + 1 μg free pL. After a further 30 minutes room temperature incubation the mixture was added to the cells. After a 2 hour incubation at 37°C, 4 ml of DMEM + 10 % FCS was added to each dish. 24 hours later cells were harvested and luciferase activity was measured. Results are shown in Fig. 31.

Example 22

Gene transfer to primary airway epithelial cells

Initial studies into genetic correction of cystic fibrosis demonstrated that immortalized cell lines derived from human airway epithelium exhibited susceptibility to this gene transfer method. To exclude the possibility that this phenomenon was the result of immortalization-induced alterations of the airway epithelium, transferrin-polylysine molecular conjugates were also evaluated in human primary respiratory epithelium cells (1AE).

1AE cells were obtained from nasal polyp specimens of patients as described by Yankaskas, J.R. et al., 1987. The tissues are rinsed in sterile saline, then in Joklik's Minimum Essential Medium (MEM) plus antibiotics

(penicillin 50 U/ml, streptomycin 50 µg/ml, gentamicin 40 µg/ml) and transported to the laboratory at 4°C. Cartilage and excess submucosal tissue are dissected free, and the epithelial sheets are incubated in protease solution (Sigma, type 14, 0.1 mg/dl) in MEM at 4°C for 16 to 48 hours (Wu, R, 1985). 10% FBS (fetal bovine serum) is added to neutralize the protease, and cells are detached by gentle agitation. The resulting suspension is filtered through 10 µm nylon mesh to remove debris, pelleted (150 x g, 5 min) and washed in F12 + 10% FBS.

The cells were then treated with transferrin-polylysine conjugates (hTfpL) containing a luciferase encoding plasmid (pRSVL) as a reporter gene. In this analysis, the primary cells did not exhibit the susceptibility to these complexes exhibited by the corresponding immortalized cell lines (background = 429 light units; with the addition of conjugates: 543 light units) likely indicating a relative paucity of transferrin receptors on 1AE.

To exploit an alternative target receptor on the cells, biotinylated adenoviruses were used (cf. Example 19). The cells treated with this conjugate exhibited levels of expression significantly greater than background (2585753 ± 453585 light units). In addition, primary airway epithelial cells derived from other species also exhibited a high level of susceptibility to gene transfer by this route (mouse = 3230244 ± 343153; monkey = 53498880 ± 869481 light units).

Example 23

Gene transfer to hepatocytes and blood cells

In the experiments of this Example, the following materials and methods were used:

Transfection of tissue culture cells:

Cells of the cell line BNL CL.2 were grown as described in Example 6. HeLa cells and hepatocytes were grown in 6 cm petri dishes. Transfection was carried out at a cell density of approximately 3 x 10^5 cells per dish. Prior to transfection, 1 ml of fresh medium containing 2% FCS replaced the standard culture medium.

Formation of binary complexes:

Biotinylated adenoviruses (approx. 10^9 PFUs; see Example 19 a) and 19 b)) were reacted with 800 ng streptavidinylated polylysine in 50 µl HBS. After 30 min at room temperature, 6 µg pCMVL-DNA in 170 µl HBS were added, incubated for 30 min and then 3 µg pL300 in 200 µl HBS was added and after further 30 min the solution was used for transfection experiments.

Formation of Ternary complexes:

Biotinylated adenoviruses (approx. 10^9 PFUs) were mixed with 800 ng streptavidinylated polylysine in 50 µl HBS. After 30 min at room temperature the solution was mixed with 6 µg plasmid DNA in 170 ml HBS, incubated for 30 min, then 10 µg TfpL 1908 in 200 µl HBS was added and after further 30 min the solution was used for transfection experiments.

d) ß-galactosidase assay

BNL CL.2 cells were seeded on to cover slips and 24 h later the cells were transfected with the pCMV-ß gal (Lin and Chae, 1989) reporter plasmid. 48 h later, the ß-galactosidase assay was carried out as in Example 15.

a) Linkage between DNA condensates and adenovirus greatly enhances luciferase reporter gene expression:

The effects of the transfer of DNA into hepatocytes by means of binary and ternary DNA complexes is shown in Fig. 32: the gene transfer efficiency is augmented in

the presence of free adenovirus. The slots pLAdenoV/TpfL show the results of transfection with adenovirus which has been conjugated with polylysine by means of transglutaminase and then reacted with DNA neutralizing part of the negative charges of the DNA. Later, transferrin-polylysine has been added neutralizing the remainder of the charges. In this way, a ternary complex of adenovirus-polylysine/transferrin-polylysine/DNA was synthesized. As can be seen, an extraordinarily high value of 1.5×10^9 light units was obtained (or approx. 5000 light units per cell). In slot adenoV + pL + TfpL, adenovirus and polylysine were mixed as for the transglutaminase treatment. However, to demonstrate the specificity of the transglutaminase mediated binding of polylysine to the virus, the enzyme was omitted. Then the virus preparation was complexed to the same amount of DNA and TfpL as in pLAdenoV/TfpL. In this case, the transfection was moderate as in AdenoV + TfpL because in both experiments co-localization of virus and transferrin DNA is a stochastic process, in contrast to the experiment shown in slot pLAdenoV/TfpL where co-internalization by the linkage of virus and DNA in the ternary complex yield a high level of transferrinfection (transfection with transferrin).

b) Transfection of K562 cells reveals the endosomolytic properties of adenovirus.

The human erythroleukemic cell line K562 contains ca. 150,000 transferrin receptors (Klausner et al., 1983b). In the presence of chloroquine, these cells can be transfected at very high levels with TfpL/reporter DNA complexes even in the absence of adenovirus (TfpL, Figure 33) as shown by Cotten et al., 1990. The same complexes with added free adenovirus, but in the absence of chloroquine, yield relatively poor levels of reporter gene expression (AdenoV/TfpL) presumably because K562 cells like other blood cells (Silver et al., 1988;

Horvath, et al., 1988) have low levels of adenovirus receptors. When the adenovirus is linked to polylysine via a biotin/streptavidin bridge and the reporter DNA is fully condensed by addition of more polylysine to complete the binary complex (pLAdenoV/pL), adenovirus supported transfection reaches intermediate levels, presumably because the few adenovirus receptors on K562 cells are used efficiently. If however the complexed adenovirus-polylysine-DNA is fully condensed and neutralized by addition of polylysine-transferrin to form a ternary complex (pLAdenoV/TfpL) and the numerous cellular transferrin receptors come into play, the transfection efficiency, owing to both efficient transferrin binding and to the endosomolytic properties of the virus, is increased by at least another two orders of magnitudes (Figure 33).

c) Ternary DNA complexes lead to the expression of the reporter gene in almost 100% of hepatocytes.

To test the efficacy of the transport system in mouse hepatocytes (BNL CL.2), the cells were transfected with the ß-galactosidase reporter gene. Figure 34 shows the ß-galactosidase assay after a) transferrinfection in the presence of chloroquine; b) transferrinfection in the presence of free d1312 adenovirus and c) transfection with ternary, (d1312) adenovirus-polylysine-transferrin-DNA complexes. In the absence of adenovirus, after standard transferrinfection of the reporter DNA, only few cells express the reporter gene. The percentage of transfection is less than 0.1%. When chloroquine is included the percentage is increased to about 0.2% (Figure 34A). With free adenovirus about 5 - 10% of the cells express the reporter gene (Figure 34B) while the ternary complexes with transglutaminase modified virus lead to expression in most, if not all, cells (Figure 34C). Because the ternary complexes can

be used at high dilution, the toxic effect seen with high doses of free (inactivated) adenovirus does not usually arise. But it should be noted that where ternary complexes are deployed at high concentration in order to reach 100% of the tissue culture cells, a similar toxic effect becomes noticeable. The toxic effects may be caused by residual viral gene activity, the endosomolytic properties of the added virus or is simply a consequence of the very high level of expression of the transfected gene.

d) Expression of a transfected reporter gene is transient but lasts for weeks in non-dividing hepatocytes.

Ternary transport complexes (pLAdenoV/TfpL) were made with polylysine-adenovirus and modified adenovirus further inactivated by reacting the virus with psoralen. A 2/3 confluent hepatocyte cell culture was transfected as in Figure 34B with the luciferase reporter gene plasmid CMVL and luciferase activity was determined at different time points. As can be seen from Figure 35, luciferase activity was maximal after 3 days at which time the hepatocyte cell culture became confluent and the cells stopped dividing. Expression of the reporter gene persisted in the non-dividing cell culture without applying selection for the maintenance of the gene and lasted for at least 6 weeks, especially when psoralen inactivated adenovirus was used for the formation of the ternary complexes.

Example 24

The Use of the Chicken Adenovirus CELO to Augment DNA Delivery to Human Cells

In this example, the chicken adenovirus CELO was tested for its ability to augment DNA delivery into

human HeLa cells in a fashion analogous to the above experiments employing the human adenovirus Type 5.

The chicken adenovirus CELO (Phelps strain, serotype FAV-1,7, chicken kidney cell passage) was used in these experiments. The virus (2 ml) was passed through a PD-10 gel filtration column equilibrated with 20 mM HEPES pH 7.3, 150 mM NaCl, (HBS) + 10% glycerol and 2 ml of the eluent was reacted with 20 µl 1 mM NHS-LC-biotin (Pierce) for 3 hours at room temperature. The biotinylated virus was subsequently dialyzed against 3 x 300 ml of HBS + 40% glycerol at 4°C and subsequently stored in aliquots at -70°C.

HeLa cells (5 x 10^5 cells per 6 cm dish) were incubated in 2 ml of DMEM + 2% FCS with 6 µg of the plasmid pCMVL complexed with polylysine (pLys) or transferrin-polylysine (TfpL) mixtures in 500 µl HBS (the complexes were pre-incubated for 30 minutes at room temperature). The samples were then added to the cells in the presence of the quantity of virus indicated in Figure 36. With the samples containing biotinylated CELO virus, the indicated quantity of virus was preincubated with the indicated quantity of streptavidin-polylysine (StrpL) in 200 µl HBS for 30 minutes at room temperature before adding 6 µg of the plasmid pCMVL in 100 µl HBS. After a 30 minute room temperature incubation, the indicated quantity of TfpL material was added to the cells at 37°C. Two hours later, 5 ml of DMEM + 10% FCS was added to the cells and 24 hours later the cells were harvested and processed for luciferase assay.

As shown in Figure 36, the CELO virus in free form augmented DNA delivery into HeLa cells (lanes 1-6). However, when the CELO virus was modified with biotin and included in a complex with streptavidin, either with or without additional transferrin-polylysine the virus was found to augment DNA delivery at a level that is comparable to that achieved with the human

adenovirus dl312. The particular line of HeLa cells displays a high binding capacity for polylysine/DNA complexes in the absence of transferrin (compare the luciferase activity of samples 1 and 4 in Figure 36). Thus, the inclusion of the CELO virus in a polylysine DNA complex is sufficient to trigger uptake of the virus.

Example 25

Transfection of myoblasts

a) Transfection of myoblasts and myotubes with DNA/transferrin-polylysine complexes in the presence of free adenovirus and in the presence of biotin/streptavidin-coupled adenovirus

C2C12 myoblasts (Blau et al., 1985; ATCC No.: CRL 1772) and G8 myoblasts (ATCC No.: CRL 1456) were grown in high glucose DMEM plus 10% FCS. Myoblast cultures were transfected at subconfluence with ca. 5 x 10^5 cells per 6 cm dish. Myotube cultures were prepared by plating myoblasts in 6 cm dishes (ca. 5 x 10^5 cells per dish) and changing the medium to high glucose DMEM plus 2% horse serum when the cells reach confluence (Barr and Leiden, 1991; Dhawan et al., 1991). Myotube transfections were performed 5-7 days later. The transfection complexes were prepared as described in Example 19 using the indicated quantities of TfpL, StrpL and biotinylated adenovirus dl312. The cells were harvested 20 hours post-transfection and processed for luciferase activity measurement. Fig. 37 indicates the resulting luciferase activity for the entire cell sample. Both myoblast and myotube cultures could be transfected with high efficiency. Upon differentiation to myotubes there was less than one log decrease in transfection efficiencies (C2C12) or no significant

decrease (G8). The participation in myotube formation occurred at a lower frequency with the G8 cell line which may partly account for the lack of a detectable decrease in efficiences in the differentiated culture. The role of the transferrin/transferrin receptor interaction in the DNA delivery to this type of cell was not major. In all four cell preparations there was only weak delivery of DNA using TfpL/DNA complexes in the presence of free adenovirus dl312 (lanes 1,4,7,10). Transfection efficiencies were enhanced using the coupled virus system (lanes 2,3,5,6,8,9,11,12). There was a less than 1 log increase in efficiencies comparing the gene transfer obtained with combination complexes containing only virus and polylysine/StrpL to the results obtained with complexes which include transferrin-polylysine (compare, for example, lane 2, no transferrin, with lane 3, transferrin). The poor transfection with free virus and the high transfection with coupled virus complexes either in the presence or absence of transferrin-polylysine suggest that the adenovirus serves as the ligand in these cells and in the absence of coupling, the free virus may enter cells but the TfpL/DNA complex does not enter productively. (The DNA used in this Example was pCMVL, designated pCluc in the Figure.)

b) Histochemical analysis of transfection frequencies in myotubes

C2C12 myotube cultures ($5x10^5$ cells, as myoblasts, seeded per 6 cm dish and differentiated into myotubes) were prepared as described in a). With the free virus samples, pCMVß-gal DNA (6µg) was complexed with 8 µg TfpL in 500 µl HBS and supplied to the cells in the presence of 18 µl of adenovirus dl312 ($1x10^{12}$ virus per ml) in 2 ml of DMEM/2% FCS. Coupled virus samples were prepared with pCMVLacZ DNA (6µg) complexed with 7µg TfpL and 800 ng of StrpL plus 18 µl of biotinylated

adenovirus dl312 ($1x10^{12}$ virus per ml) in 500 µl HBS and supplied to cells in 2 ml of DMEM/2% FCS. After a 24 hour incubation cells were stained for β-galactosidase activity, as described in Example 15.

The β-galactosidase staining patterns were consistent with the results of transfections using luciferase as the reporter gene (see a). Very low gene expression was obtained in myotube cultures using the free virus while coupling the virus and DNA result in high level gene expression. The presence of blue-stained, multi-nucleated tubules indicated the successful transfer of a gene to these differentiated cells in the presence of free adenovirus.

c) Delivery of DNA to mouse primary myoblast and myotube cultures

The major skeletal muscles from both hind legs of a 4 week-old male C57Bl/6 mouse were sterilely isolated into PBS and minced into approximately 5 mm pieces. The tissue was suspended in 20 ml of PBS, allowed to settle for ca. 2 minutes and the supernatant was aspirated. This washing was repeated 3 times. The tissue was then mixed with 3.5 ml of PBS plus 0.5 ml of trypsin/EDTA, 0.5 ml of 1% (w/v) collagenase (type 2, Sigma), and 0.5 ml of 1% BSA (fraction V, in 4 mM $CaCl_2$) and allowed to incubate at 37°C for 30 minutes with frequent, gentle agitation. At the end of the 30 minute incubation the remaining tissue was allowed to settle and the supernatant was removed and mixed with 5 ml of DMEM +20% FCS. The incubation with protease was repeated 3-4 times until the tissue was completely dispersed. The cell suspension was then passed through a cell sieve (Falcon) to remove any aggregates and tissue fragments, and centrifuged at 500g for 15 minutes. The cell pellet was resuspended in 10 ml of DMEM+20%FCS and the fibroblasts were removed by plating the cells on a 15 cm diameter, uncoated tissue culture dish for

60 minutes. The unattached cells were then carefully removed and plated on five laminin-coated, 10 cm tissue culture dishes with 15 ml of DMEM,+20%FCS per dish. Upon reaching confluence (approximately one week later) the cells were trypsinized and replated on laminin-coated, 6 cm dishes, approximately 1x 10^6 cells per dish. To generate myotube cultures, approximately 5 days later (when the cells had reached confluence) the medium was changed to DMEM+2% horse serum and one week later transfections were performed. Myoblast cultures for transfection were transfected in 6 cm dishes at approximately 80% confluence. Laminin-coated cell culture plates were prepared in the following manner. Cell culture dishes were coated with 0.025 mg/ml polylysine (Mw 30,000-70,000, Sigma) in sterile water for 30 minutes at room temperature. The plates were rinsed 3 times with sterile water and air dried. The plates were then coated with 8 µg/ml laminin (EHS, Sigma) in water overnight at room temperature. Plates were then washed 3 times with sterile water before seeding cells.

The DNA complexes used for transfections were prepared by diluting the indicated quantity of psoralen/UV-inactivated biotinylated adenovirus dl312 (prepared as described in Example 19) in 150 µl of HBS and adding 1 µg of StrpL in 150 µl of HBS followed by a 30 minute, room temperature incubation. HBS (100 µl) containing 6 µg of pCMVL (designated pCluc in the Figure) was then added to each sample followed by another 30 minute room temperature incubation. Finally, 7 µg of TfpL in 100 µl of HBS was added to each sample, incubated for 30 minutes at room temperature and then supplied to either myoblast or myotube cultures in 6 cm dishes containing 2 ml of DMEM +2% FCS. After a 1 hour incubation, the medium was replaced with 5 ml of DMEM+20%FCS (myoblasts) or DMEM+2% horse serum (myotubes) and the cells were harvested for luciferase

analysis 48 hours later. The luciferase activity from the entire cell sample is displayed in Fig. 38.

Example 26

Improvement of CELO virus delivery to myoblasts using a lectin ligand

a) Comparative analysis of Adenovirus dl312 and CELO virus in HeLa cells and C2C12 myoblasts

Samples of either HeLa cells or C2C12 myoblasts (5×10^5 cells per 6 cm dish) were transfected with 6 µg pCMVL (designated pCluc in the Figure) complexed with 1 µg StrpL/7µg TfpL plus 5 µl of biotinylated Adenovirus dl312 (see Example 19, 1×10^{12} particles/ml) or 18 µl of biotinylated CELO virus (see Example 24, 0.3×10^{12} particles per ml). After a 20 hour incubation the cells were harvested and processed for luciferase activity measurement. Fig. 39 indicates the resulting luciferase activity from each entire cell sample.

Transfection into HeLa cells could be performed with comparable efficiency using either the human adenovirus dl312/StrpL/TfpL/DNA complexes, which can enter the cells by either the adenovirus receptor or the transferrin receptor, or the CELO virus/StrpL/TfpL/DNA complexes which can enter via the transferrin receptor. However, while delivery of DNA into C2C12 myoblasts could be performed efficiently with adenovirus dl312 complexes, complexes containing the CELO virus functioned poorly in these cells. Previous examples have demonstrated that the transferrin receptor plays only a minor role in combination complex delivery to these cells; presumably the adenovirus receptor is the major site of entry. The poor activity of the CELO virus in myoblasts might then be due to a poor binding of both the CELO virus and transferrin to the C2C12 myoblasts.

b) Improvement of CELO virus C2C12 myoblast transfection using wheat germ agglutinin as a ligand

Due to the poor delivery obtained in a), a new ligand was selected to replace transferrin, namely biotinylated wheat germ agglutinin (2-4 moles biotin per mole of protein; Boehringer Mannheim). Biotinylated CELO virus was prepared as previosuly described. Complexes containing 6 µg pCMVL plus the indicated quantities of StrpL, TfpL, biotinylated wheat germ agglutinin (WGA-B) and CELO virus were prepared in the following manner. Virus and WGA were diluted, together, in 150 µl HBS. StrpL was also diluted in 150 µl HBS and the two solutions were mixed and incubated at room temperature for 30 minutes. The DNA, diluted in 100 µl of HBS, was added to the StrpL/Virus/WGA solution followed by another 30 minute room temperature incubation. Finally, TfpL in 100 µl HBS was added to the mixture and again the sample was incubated at room temperature for 30 minutes. The complexes were supplied to C2C12 myoblasts (5 x 10^5 cells per 6 cm dish) in 2 ml of DMEM plus 2%FCS. One hour later 5 mls of DMEM plus 10% FCS was added to the cells and 20 hours later the cells were processed for luciferase activity measurement. The activity (light units) in each entire cell sample is displayed in Fig. 40. (The DNA used in this Example was pCMVL, designated pCluc in the Figure.)

Very poor DNA delivery was obtained in the absence of virus either with or without the WGA (lanes 1,6). Moderate delivery was obtained with coupled CELO virus (lane 2); however a 16-fold increase in delivery was obtained if WGA-B is included in the complex. Increasing the quantity of WGA in the complex (from 1 µg to 5 µg) resulted in a slight decrease in delivery (compare lanes 3 and 4) while increasing the StrpL content of the complex (from 1 µg to 2 µg) enhanced the

delivery slightly (compare lanes 3 and 5). These results clearly indicate that WGA-B as a ligand enhances CELO virus-mediated DNA delivery to C2C12 cells.

d) Expression of a full length factor VIII gene in C2C12 myoblast and myotube cultures

The myoblast and myotube cultures were prepared as described above. Transfections were performed using 6 µg of a plasmid containing a full-length factor VIII cDNA (Wood et al., 1984; Eaton et al., 1986) complexed with 5 or 15 µl of biotinylated adenovirus (as indicated) plus 0.5 or 1 µg StrpL, and 7 or 6 µg of TfpL in the standard complex formation protocol.

The DNA/virus complexes were supplied to cells in 2% FCS/DMEM. After a 4 hour incubation at 37°C, 3 ml of fresh DMEM+10 % FCS was added to each dish. 18 hours later the medium was harvested and assayed for the presence of factor VIII using a COATEST, (KABI, Pharmacia) test system with an international standard as a reference. Factor VIII levels are plotted as mUnits generated per 24 hours, per 1 x 10^6 cells (Fig. 41).

Example 27

Use of adenovirus protein for DNA delivery

Adenovirus (wild type 300) was grown in HeLa cells, purified and biotinylated as described for adenovirus dl312. 1.2 ml of virus was dialyzed against 3 x 300 ml of 5 mM MES (2-(N-morpholino)ethanesulphonic acid), 1 mM EDTA pH 6.25, 4°C, for 18 hours. The material was then centrifuged for 30 minutes at 27 K in an SW60 rotor. The supernatant was carefully removed, the pellet was resuspended in HBS/40% glycerol. HEPES, pH 7.4 and NaCl were added to the supernatant to 20 mM and 150 mM, and both the pellet (containing the viral core protein and the bulk of the hexon capsid, "core" in Fig. 42) and the

supernatant fractions (containing the vertices, "vertices" in Fig. 42) were tested for DNA delivery activity into both Mov13 mouse fibroblasts (Strauss and Jaenisch, 1992) or HeLa cells.

Complex formation with DNA was performed in the following manner. The indicated quantities of each fraction, disrupted virus before centrifugation or intact virus (expressed as µg protein as determined by a Bradford assay) were diluted in 300 µl HBS. Streptavidin-polylysyine (3 µg in 50 µl HBS) was then added followed by a 30 minute room temperature incubation. 6 µg pCMVL, designated pCluc in the Figure, was diluted in 100 µl HBS and added to the first solution for a 30 minute incubation. Finally, 2 µg of TfpL in 100 µl of HBS was added followed by another 30 minute incubation. In samples prepared with only TfpL, 8 µg of TfpL in 170 µl HBS was mixed with 6 µg of pCMVL in 330 µl of HBS for 30 minutes at room temperature. The indicated quantities of virus protein were diluted into 300 µl of HBS and then added to the TfpL/DNA complexes. All samples were then added to 5 x 10^5 cells in 6 cm dish, containing 2 ml of DMEM/10%FCS (either HeLa of Mov13 fibroblasts) for 1 hour. 5 ml of fresh medium containing 10% FCS was then added and the cells were processed for luciferase activity 20 hours later. The resulting luciferase activity (in light units) is displayed in Fig. 42 for both Hela cells (panel A) or Mov13 fibroblasts (panel B).

With both cell types there is a dose-dependent increase in DNA delivery activity associated with the vertex fraction (sample 4-6 in both panels). When the same quantity of biotinylated virus protein is included with TfpL/DNA complexes lacking streptavidin-polylysine DNA delivery close to background levels is observed (sample 3 in each panel).

Example 28

Enhanced gene transfer using DNA ternary complexes containing galactose-ligand conjugate

a) Ternary complexes containing influenza peptide conjugate

The presence of polylysine-conjugated peptides containing sequences derived from the N-terminus of influenza virus hemagglutinin HA-2 subunit, in DNA/transferrin-polylysine complexes has been found to substantially augment the transferrin-polylysine mediated gene transfer (Examples 13 and 14).

Similar DNA combination complexes containing the tetra-antennary galactose ligand-polylysine conjugate and the polylysine-modified influenza peptide (prepared as described in Example 6 or 13, respectively) have been prepared by adding the ligand-polylysine conjugate to plasmid DNA pCMVL to neutralize half of the DNA charge, the remainder of the charge being used to load the complexes with influenza peptide-polylysine conjugate. The delivery of these DNA complexes, containing the synthetic ligand (gal)4, to BNL CL.2 hepatocytes (transfections were carried out as described in Example 6 g) resulted in a luciferase gene expression (Fig. 43) that was significantly higher than the expression obtained with transferrin as ligand. The expression was more than 500 times higher than in control experiments obtained with DNA complexes lacking the influenza peptides, but containing the same amount of polylysine (Fig. 43). The activity obtained with the DNA combination complexes was also approx. 30 times higher than with DNA/(gal)4pL complexes incubated with cells in the presence of chloroquine.

b) Ternary complexes containing adenovirus conjugate

Complexes were prepared as follows: biotinylated

adenovirus dl312 (prepared as in Example 19; 2µl, 6µl or 18µl; 10^{12} particles/ml) in 50 µl HBS were mixed with streptavidin-polylysine (100 ng, 160 ng, or 480 ng) in 100 µl HBS. After a 30 min incubation, a solution of 6 µg pCMVL in 200µl HBS was added, and after further 30 min, a solution of 3.8µg (gal)4pL (prepared as in Example 6) or 7µg TfpL in 150µl HBS was added. The DNA complex solutions were added to each 300,000 cells (ATCC TIB73, ATCC TIB74, ATCC TIB75, ATCC TIB76) grown in 6 cm plates in high glucose DMEM + 2%FCS. Further cell culture procedures and luciferase assays were performed as described in the preceding Examples. Gene expression (after 24h) as shown in Fig. 44.

Example 29

Gene transfer into B-lymphoblastoid B-cells

Human Ig- and anti-human-Ig-polylysine conjugates were prepared as follows, the coupling being carried out using methods known from the literature by inserting disulphide bridges after modification with succinimidyl-pyridyldithiopropionate (SPDP, Jung et al., 1981):

a) Preparation of anti-human-Ig-polylysine 300 conjugates

A solution of 2 mg of goat-anti-human-Ig (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) in HBS (150 M NaCl, 20 mM HEPES, pH 7.8) was mixed with 14 µl of a 5 mM ethanolic solution of SPDP (Pharmacia). After 10 h at ambient temperature the solution was filtered over a Sephadex G25 gel column (eluant 100 mM HEPES buffer pH 7.3), to obtain 1.3 mg of anti-human-Ig, modified with 30 nMol of pyridyldithiopropionate residues. Poly(L)lysine 300 (average polymerisation level of 300 lysine groups (Sig. a)), was modified analogously with SPDP and brought into the form modified

with free mercapto groups by treating with dithiothreitol and subsequent gel filtration. A solution of 12 nMol of polylysine 300, modified with 29 nMol of mercapto groups, in 0.3 ml of HBS was mixed with the above-mentioned modified anti-human-Ig, with the exclusion of oxygen, and left to stand overnight at ambient temperature. The reaction mixture was adjusted to a content of 0.6 M NaCl by the addition of 5 M NaCl. The conjugates were isolated by ion exchange chromatography (Pharmacia, Mono S HR 5/5); after dialysis against 25 mM HEPES pH 7.3, corresponding conjugates were obtained consisting of 0.33 mg of anti-human-Ig, modified with 4 nMol of polylysine 300 (molar ratio 1:2).

b) Preparation of human Ig-polylysine 300 conjugates

A solution of 19.5 mg (122 nMol) of antibody (Sigma I-4506) in 2 ml of HBS was combined with 39 µl of 10 mM ethanolic solution of succinimidyl-pyridyldithio-propionate (SPDP, Pharmacia). After 2.5 h at room temperature the mixture was filtered over a Sephadex G 25 gel column (eluant 100 mM HEPES buffer pH 7.9), to obtain 19 mg (119 nMol) of human-IgG, modified with 252 nMol of pyridyldithiopropionate groups. Poly-L-lysine 300 (average polymerisation level of 300 lysine groups; Sigma) was modified analogously with SPDP and brought into the form modified with free mercapto groups by treating with dithiothreitol and subsequent gel filtration. A solution of 119 nMol of polylysine 300, modified with 282 nMol of mercapto groups, in 1 ml of HBS was mixed with the above-mentioned modified human-Ig under the exclusion of oxygen and left to stand overnight at ambient temperature. The reaction mixture was adjusted to a content of about 0.6 M NaCl by the addition of 5 M NaCl. The conjugates were isolated by ion exchange chromatography (Mono S, Pharmacia, 50 mM

HEPES pH 7.3, salt gradient 0.6 M to 3 M NaCl); after dialysis against HBS pH 7.3, corresponding conjugates were obtained consisting of 9 mg (57 nMol) of antibody, modified with 90 nMol of polylysine 300 (molar ratio 1:1.6).

c) Complexing and transfection

The complexes were prepared as follows:

Biotinylated adenovirus d1312 (30 µl, 10^{12} particles per ml) in 50 µl of HBS were mixed with streptavidin-polylysine (800 ng) in 100 µl of HBS: after 30 minutes incubation a solution of 9 µg pCMVL in 200 µl of HBS was added. After a further 30 minutes a solution of 5.1 µg of polylysine (pLys450), 10.2 µg of TfpL, 12 µg of human-IgG-polylysine conjugate or 10 µg of anti-human-Ig-polylysine conjugate in 150 µl of HBS was added. The DNA complexes were each added to 10^6 B-lymphoblastoid cells (the cells were obtained from human peripheral mononuclear blood cells by immortalising with Epstein Barr virus as described by Walls and Crawford, 1989, and cultured in 1 ml of RPMI 1640 + 2% FCS in 24-well dishes). The further progress of the cell culture and luciferase assays were as described for the preceding Examples. The gene expression values obtained (luciferase activity in light units) are given in Fig. 45.

Example 30

DNA transfer with transferrin-polylysine in the presence of free and conjugated rhinovirus

a) Rhinovirus HRV-2 preparations

Rhinovirus HRV-2 was prepared and purified as described by Skern et al., 1984.

A 400 µl solution of rhinovirus (approx. 30µg) in

HBS (150 mM NaCl / 5 mM HEPES, pH 7.9) / 10% glycerol was treated with 10 nmol of NHS-LC-biotin (Pierce 21335). After incubation for 3 hrs at room temperature, the virus was separated from unincorporated biotin by extensive dialysis against HBS/ 40% glycerol at 4°C.

Light-sensitive rhinovirus, prepared by growing the virus in the presence of acridine orange, was inactivated as described by Madshus et al., 1984.

b) Preparation of DNA complexes and transfections

i) Transferrin-polylysine / DNA complexes were prepared by mixing a solution of 6 µg of plasmid DNA pCMVL in 330 µl HBS (150 mM NaCl, 20 mM HEPES, pH 7.3) with a solution of 8 µg TfpL290 in 170 µl HBS. DNA complexes were mixed with 1.5 ml of medium (DMEM plus 2%FCS) and with 0.14 µg to 3.5 µg rhinovirus HRV-2 (or inactivated HRV-2). The mixture was added to NIH 3T3 cells (300,000 cells per 6 cm plate). Four hours later the transfection medium was replaced by 4 ml of fresh medium (DMEM plus 10% FCS). Cells were harvested after 24 hrs and assayed for luciferase activity as previously described (Fig. 46A).

ii) DNA combination complexes containing transferrin-polylysine and rhinovirus-polylysine conjugates were prepared as follows: a 100-µl solution of biotinylated rhinovirus HRV-2 (3.5µg) in HBS was mixed with 1µg StrpL polylysine in 100 µl HBS. (The other virus concentrations were mixed with appropriate portions.) After 30 min at room temperature, the solution was mixed with 6 µg of plasmid DNA in 150 µl HBS, incubated for a further 30 min at room temperature, and subsequently mixed with 6µg TfpL290 in 150 µl HBS. DNA complexes were mixed with 1.5 ml of medium (DMEM plus 2%FCS) and added to NIH 3T3 cells (300,000 cells per 6 cm plate). Further treatment of the cultures and the assay for luciferase activity was performed as

described in i) (Fig. 46B).

Example 31

Transfection of HeLa cells with combination complexes containing ionically bound adenovirus

Complex formation A): DNA complexes were prepared by first mixing 30 µl adenovirus dl312 (approx. 10^9 PFUs) with 1 µg polylysine pLys450 (with an average chain length of 450 monomers) in 170 µl HBS and, after 30 min at room temperature, subsequent mixing with 6 µg of pCMVL-DNA in 170 µl HBS. After an incubation for another 30 min, complexes were mixed with 9 µg TfpL190 in 170 µl HBS. An aliquot of the complex mixture (10 % = 50 µl solution, 600 ng DNA; or 1 % = 5 µl solution, 60 ng DNA) was diluted in 1.5 ml DMEM plus 2 % FCS and added to 300,000 HeLa cells. After 4 h, 2 ml of DMEM plus 20 % FCS was added. Harvesting of cells 24 h after transfection and luciferase assay were performed as described. The luciferase activity corresponding to the total extract were 29,115,000 light units (in the case of 600 ng DNA) and 1,090,000 light units (in the case of 60 ng DNA).

Complex formation B) (Control Experiment): DNA complexes were prepared by first mixing 6 µg of pCMVL-DNA in 170 µl HBS with 1 µg polylysine pLys450 in 170 µl HBS and, after 30 min at room temperature, subsequent mixing with 9 µg TfpL190 in 170 µl HBS. After an incubation for another 30 min, complexes were mixed with 30 µl adenovirus dl312 (approx. 10^9 PFUs). An aliquot of the complex mixture (10 % = 50 µl solution, 600 ng DNA; or 1 % = 5 µl solution, 60 ng DNA) was diluted in 1.5 ml DMEM plus 2 % FCS and added to 300,000 HeLa cells. After 4 h, 2 ml of DMEM plus 20 % FCS was added. Harvesting of cells 24 h after transfection and luciferase assay were

performed in conventional manner. The luciferase activity corresponding to the total extract were 405000 light units (in the case of 600 ng DNA) and 200 light units (in the case of 60 ng DNA).

Example 32

Local application of DNA/adenovirus/transferrin-polylysine conjugates into rat liver

a) Direct injection

The DNA complexes were prepared as described in Example 19. They comprised 200 µl Adenovirus dl312, 6.4 µg streptavidin-polylysine, 48 µg pCMVL and 48 µg TfpL290 in a total volume of 2000 µl HBS. A male Sprague-Dawley rat (body weight 240g) was anaesthetised with Avertin and a laparotomy of 4 cm performed. The complex solution was injected in a time span of 2 minutes into the left lobe of the liver. Then the laparotomy wound was closed in layers. 48 hours after injection of the complexes the rat was sacrificed under ether anaesthesia and the luciferase expression in various liver samples measured. In the area of injection 5.615 light units/per mg protein of the liver homogenate were measured. Total activity was 370,600 light units. No luciferase activity was measurable in areas of the liver away from the injection site.

b) Application of conjugates to the liver via the bile draining system

The complexes were prepared as follows: 200 µl biotinylated Adenovirus dl312 diluted with 200 µl HBS were incubated with 6.4 µg streptavidin-modified polylysine in 400 µl HBS for 30 minutes at room temperature. Then 48 µg of pCMVL in 800 µl HBS were added. After 30 minutes of incubation 48 µg of TfpL in 900 µl HBS were further added. For application of the

complexes male Sprague Dawley rats (250 g body weight) were anaesthetized with Avertin and the abdomen opened with a median incision. The intestine was displaced to the left side of the body and a 27 G needle, which had been attached to a tube and a 1 ml syringe, was inserted into the bile duct. The injection of the complexes was performed over a period of 4 minutes. Then the needle was retracted from the bile duct and the injection site sealed with a fibrin sealer (Immuno). The abdominal wound was closed with sutures and metal clips. After 30 hours the rat was killed and samples from different lobes of the liver were assayed for luciferase gene expression. The peak activity of luciferase was 19000 light units/mg protein and the calculated overall expression in the total liver was in the range of 2.7×10^6 light units.

Example 33

Application of DNA/aderovirus/TfpL complexes into the clamped mouse tail vein

The complexes were prepared as described in Example 19. They comprised 45 µl Adenovirus dl312, 0.8 µg streptavidin-polylysine, 6 µg pCMVL and 24 µg mTfpL290 in a total volume of 150 µl HBS. The complexes were injected into the tail vein of a male C3H/He mouse (two months old), which had been anesthezised with Avertin. Immediately after injection the tail vein was clamped at the proximal and distal end of the tail such that the complex solution was restricted to the segment of the tail vein which had been injected during the period of compression (25 minutes) and could not be flushed by the blood. 48 hours after injection the mouse was sacrificed by cervical dislocation and the tail vein prepared. Luciferase expression was measured in the homogenate of the tail vein segment. Expression resulted in 2,600

light units/3 cm tail vein.

Example 34

Transfection of primary human melanoma cells

a) Transfection with adenovirus-polylysine/transferrin-polylysine combination complexes

Primary melanoma cells were isolated from a melanoma, which had been surgically removed from a patient. The tumor was mechanically disrupted in RPMI 1640 cell culture medium plus 5% FCS, 2mM glutamine, and antibiotics and pressing the tissue fragments through a steel sieve. The tumor cells were washed several times by centrifugation and subsequent resuspension and seeded into T25 cell culture flasks. 24 hours after isolation, the tumor cells were transfected with ternary complexes comprising 3 µl, 9 µl or 27 µl biotinylated Adenovirus dl312 ($1x10^{12}$/ml), 0.5 mg streptavidin-polylysine, 6 µg pCMVL and 7 µg TfpL290 (in admixture with 27 µl adenovirus:1 µg streptavidin-polylysine and 6 µg TfpL290) in a total volume of 500 µl HBS. 36 hours after transfection the cells were harvested and the luciferase activity was determined (light units) (see Fig. 47; the top bar shows the results with the cells in suspension, the bottom bar with the adhering cells).

b) Transfection with adenovirus-polylysine/low density lipoprotein-polylysine combination complexes

i) Preparation of the LDL-polylysine conjugates

A solution of 10 mg (14.3 nmol) of LDL (low density lipoprotein, Sigma, L-2139, molecular weight 3,500,000, particle diameter about 26 nm) in 2 ml of HBS was mixed with 143 µl of a 10 mM ethanolic solution of SPDP

(1.43 µmol; Pharmacia) and left to react for 2 hours at ambient temperature. Gel filtration over a Sephadex G25 column (14 x 140 mm) with HBS yielded 3.2 ml of a solution of about 10 mg of LDL, modified with 0.70 µmol of pyridyldithiopropionate groups. The solution was mixed with 1.2 ml of 5 M NaCl in order to prevent the precipitation which would otherwise occur when polylysine was subsequently added. Poly-L-lysine with an average chain length of 300 monomers was modified with SPDP as described and brought into the form modified with mercapto groups by treating with dithiothreitol and subsequent gel filtration. The modified LDL-solution described above was mixed under argon with a solution of 0.33 µmol of polylysine 300, modified with 0.76 µmol mercapto groups, in 4 ml of 2 M NaCl, 0.5 M HEPES, pH 7.9, and the resulting mixture was left to stand for 48 hours at room temperature. The reaction solution was diluted to 32 ml with sterile water (NaCl concentration about 0.5 M) and separated by ion exchange chromatography (Biorad Macroprep S, 10 x 100 mm, 20 MM HEPES pH 7.3, gradient from 0.2 - 3 M NaCl). The product fractions were eluted at a salt concentration of 1.8 M to 2.2 M and then pooled. After dialysis against HBS, conjugates consisting of 2.35 mg (3.4 nmol) of LDL modified with 190 nmol of polylysine (corresponding to 7.5 mg of the free polylysine base form) were obtained. This corresponds to an average modification of the LDL-particles with about 55 polylysine chains.

ii) Complexing and transfection:
18 µl of biotinylated adenovirus dl312 preparation were diluted with HBS to a volume of 100 µl. 1.2 µg of streptavidin-polylysine were adjusted to 100 µl volume using HBS and mixed with the 100 µl of adenovirus preparation. After 30 minutes 150 µl of HBS with 6 µg of pCMVL were added thereto. After a further 30

minutes, 300 µl of HBS with 4 µg of LDLpL (polylysine content 20 µg) were added thereto. The solution thus prepared was mixed with 1.5 ml of DMEM (10% FCS) and added to 3 x 10^5 primary myeloma cells (prepared as in a) and referred to as HMM1 and HMM4), which were in a cell culture dish with a diameter of 6 mm. The other cell culture work and luciferase measurements were carried out as described in a). The gene expression can be seen in Fig. 48; A) shows the results with the myeloma cells HMM1; all the experiments were carried out with AdpL-conjugates, which are not separately mentioned in the Figures. B) shows the experiments with the myeloma cells HMM4; all the experiments were carried out with AdpL-conjugates which are shown separately only in the last column (the name d1312/16 refers to the special virus preparation). In the experiments shown in column 2 LDL was added in a 25-fold excess, which resulted in a reduced gene transfer efficiency as a result of the competition for the LDL-receptor.

Example 35

Transfection of primary human fibroblasts

Human skin biopsies were put into a 6 cm petri dish containing DMEM, 2 mM glutamine, 20 % FCS and antibiotics. Then the biopsies were thoroughly minced with a surgical knife and cultured in the presence of 3 ml medium for 5 days. Thereafter the cells were washed with fresh medium and cultured for further 7 days. After this period of time the cells were trypsinized and subcultured into new petri dishes. When the cells were almost confluent, they were trypsinized again and stored frozen until transfection. For transfection the cells were thawed and seeded into 6 cm petri dishes and cultured in DMEM containing 2 mM glutamine, 10 % FCS and antibiotics. The transfection conjugates were prepared

as follows: 3 µl, 10 µl, 20 µl and 30 µl of biotinylated adenovirus dl312 were incubated with 0.1 µg, 0.3 µg, 0.5 µg and 0.8 µg polylysine-modified strepavidine in 150 µl HBS for 30 minutes at room temperature. Then 6 µg of pCMV-ßgal plasmid in 170 µl HBS were added and the mixture was incubated for a further 30 minutes. In the final step 7.8 µg TfpL for the conjugates with 3 µl dl312, 7 µg TfpL for 10 µl dl312 and 6 µg TfpL for the conjugates with 20 µl and 30 µl dl312 in 170 µg HBS were added. After an incubation period of 30 minutes the conjugates were applied to the cells in 2 ml DMEM containing 2 mM glutamine, 2 % FCS and antibiotics and the cells were incubated for 4 hours at 37°C. Then the medium was removed and culture was continued at 37°C with DMEM containing 2 mM glutamine, 10 % FCS and antibiotics. After 48 hours the expression of ß-galactosidase was demonstrated as described in previous Examples.

In the transfection with 3 µl dl312 14 % of the cells revealed production of ß-galactosidase, with 10 µl dl312 32 % positive cells were obtained, with 20 µl dl312 39 % and with 30 µl dl312 64 % of the cells were positive.

Example 36

Gene transfer into respiratory tract epithelial cells of rats in vivo by means of combination complexes

a) Preparation of TfpL/AdpL-combination complexes

Human transferrin-polylysine-DNA complexes (hTfpL) were prepared by combining 8 µg of transferrin-polylysine (Serva Biochemical) in 150 µl of HBS (150 mM NaCl/20 mM HEPES ph 7.3) with 6 µg of pCMVL-DNA in 350 µl of HBS and the mixture was incubated for 30 minutes at ambient temperature. Adenovirus-polylysine conjugates were prepared as described in Example 19 c);

psoralen/UV-inactivated adenovirus was used. The combination complexes hTfpL/AdpL were prepared as described in Example 23 c).

b) Administration of the complexes *in vivo* by intratracheal route

For these experiments the rat Sigmodon hispidus ("Cotton Rat") was used, as it has been shown to be a suitable animal model for human adenoviral lung diseases (Pacini et al., 1984). The animals were anaesthetised with methoxyfluran. After a vertical cut into the ventral side of the neck the windpipe was cut off square. The complexes (250 to 300 µl; 3 µg of plasmid-DNA) were injected directly into the windpipe, in full view, in the animals which had been positioned at an angle of 45°. At the times after the injection specified in Fig. 49 the animals were killed using CO_2 and the windpipe and lungs were harvested en bloc after *in situ* flushing with cold phosphate-buffered saline solution (PBS). For the luciferase tests the lung tissue was homogenised in extraction buffer, the lysates were standardised for their total protein content and the luciferase gene expression was measured as described. The light units specified refer to 1,250 µg of total protein, obtained from the lung lysates. The experiments were each carried out 3 to 4 times and the results are given as the mean ± SEM.

Example 37

Gene transfer using non-viral endosomolytic agents

a) Synthesis of membrane-disrupting peptides

i) Peptide synthesis:

Peptides were synthesized on an automatic synthesizer (ABI 431A) by the solid phase method using p-alkoxybenzylalcohol resin (0.97 mmol/g) as solid

support and fmoc-protected amino acids. The carboxy-terminal amino acid was coupled to the resin via the symmetric anhydride. Subsequent amino acids were coupled by the 1-hydroxybenzotriazole dicyclohexylcarbodiimide method. The following side chain protecting groups were used: (Trt)Asn, (Trt)Cys [(t-Bu)Cys in case of EALA and GLF], (t-Bu)Glu, (Trt)His, (t-Bu)Ser.

The peptides had the following sequences:

EALA: (SEQ ID NO:5) Trp Glu Ala Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu His Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys

GLF (SEQ ID NO:6) Gly Leu Phe Gly Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu His Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys

GLF-II (SEQ ID NO:7) Gly Leu Phe Gly Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys

GLF-delta (SEQ ID NO:8) Gly Leu Phe Glu Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys

EALA-Inf (SEQ ID NO:9) Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp Glu Gly Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys

EALA-P50 (SEQ ID NO:10) Gly Leu Phe Glu Ala Ile Glu Gly Phe Ile Glu Asn Gly Trp Glu Gly Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys

P50 (SEQ ID NO:11) Gly Leu Phe Glu Ala Ile Glu Gly Phe Ile Glu Asn Gly Trp Glu Gly Met Ile Asp Gly Gly Gly Cys

The peptides were cleaved from the resin and the side chain protecting groups were removed with the exception of (t-Bu)Cys by treatment of 100 mg peptide-loaded support with 3 ml of a mixture phenol/ethanedithiol /thioanisol/water/trifluoroacetic acid 0.75:0.25:0.5:0.5:10 for 1.5 h at room temperature. The crude peptides were precipitated in ether and washed twice. The S-t-Bu protected peptides EALA and GLF were dissolved in a small volume 1M triethylammonium bicarbonate (TEAB) pH 8, diluted to 100 mM TEAB and further purified by reverse phase HPLC on a Nucleosil 500-5C4 column (0.1% TFA - acetonitrile gradient). Both peptides eluted at about 50% acetonitrile. The free Cys-SH form of the peptides was obtained by deprotecting Trt-Cys peptides in the same way as described above. The crude peptides (5 mg) were dissolved in 100 µl 100 mM TEAB, pH 8, containing 1 µl ß-mercaptoethanol and purified by gel filtration (Sephadex G-25, 100 mM TEAB, 0.5 mM EDTA) and freeze drying or ion-exchange chromatography (Mono Q Pharmacia, 20 mM Hepes, pH 7.3, gradient 0 to 3 M NaCl, the peptide elutes at 1.5 M NaCl).

ii) Modification with N-(hydroxyethyl)maleimide

The C-terminal SH group of the peptides GLF-delta, GLF-II, EALA-Inf, EALA-P50, P50 was blocked after gel filtration of the free SH form (Sephadex G-25, 20 mM Hepes, pH 7.3, 0.5 mM EDTA) by reaction with a 1.3- to 10-fold molar excess of N-(hydroxyethyl)maleimide (1 h, room temperature). Excess maleimide was removed by gel filtration (Sephadex G-25, 100 mM TEAB, pH 8) and the peptides (GLF-delta-mal, GLF-II-mal, EALA-Inf-mal, EALA-P50-mal, P50-mal) were obtained as triethylammonium salt upon freeze drying.

iii) Modification with 2,2'-dithiobispyridine:

The free SH peptides were reacted with 10 equivalents of 2,2'-dithiobispyridine (20 mM Hepes, pH 7.9, 0.5 mM EDTA) over night at room temperature. Excess reagent was removed by gel filtration (Sephadex

G-25, 100 mM TEAB, pH 8) or ion-exchange chromatography (Mono Q Pharmacia, 20 mM Hepes, pH 7.3, gradient 0 to 3 M NaCl: the peptide elutes at 1.5 M NaCl) to obtain the (2-pyridylthio)-Cys peptides (GLF-delta-SSPy, GLF-II-SSPy, EALA-Inf-SSPy, EALA-P50-SSPy, P50-SSPy).

iv) Dimerization of peptides:

The homodimer of P50 (P50 dim) was prepared by reacting equimolar amounts of P50-Cys-(2-pyridylthio) and P50-Cys-SH in 20mM Hepes, pH 7.3, over three days at room temperature. The reaction mixture was separated on a Mono Q column (HR-5/5 Pharmacia; 20mM Hepes, pH 7.3, gradient 0.09M to 3M NaCl; P50-dimer was eluted at 1.1M NaCl). The heterodimer GLF-SS-P50 was prepared analogously by reaction of peptide P50 (free mercapto form) with pyridylthio-modified peptide GLF.

b) Liposome leakage assay:

The ability of the synthetic peptides to disrupt liposomes was assayed by the release of fluorescent dye from liposomes loaded with a self-quenching concentration of calcein. Liposomes were prepared by reverse phase evaporation (Szoka and Papahadjopoulos, 1978) with an aqueous phase of 100 mM calcein, 375 mM Na^+, 50 mM NaCl, pH 7.3 and extruded through a 100 nm polycarbonate filter (MacDonald et al., 1991) to obtain a uniform size distribution. The liposomes were separated from unincorporated material by gel filtration on Sephadex G-25 with an iso-osmotic buffer (200 mM NaCl, 25 mM Hepes, pH 7.3). For the leakage assay at various pH values, the liposome stock solution was diluted (6 µl/ml) in 2x assay buffer (400 mM NaCl, 40 m' Na citrate). An aliquot of 100 µl was added to 80 µl of a serial dilution of the peptide in water in a 96-well microtiter plate (final lipid concentration: 25 µM) and assayed for calcein fluorescence at 600 nm (excitation 490 nm) on a microtiter-plate fluorescence photometer after 30 min of incubation at room temperature. The

value for 100% leakage was obtained by addition of 1 µl of a 10% Triton X-100 solution. The leakage units were calculated as reciprocal value of the peptide concentration, where 50% leakage was observed (i.e. the volume in µl of liposome solution which is lysed to 50% per µg of peptide). Values below 20 units are extrapolated. The results of the liposome leakage assay are shown in Fig. 50. GLF and EALA exhibited the highest pH specific activity.

c) Erythrocyte lysis assay:

Fresh human erythrocytes were washed with HBS several times and resuspended in 2x assay buffer at the appropriate pH (300 mM NaCl, 30 mM Na citrate) at a concentration of 6.6 10^7/ml. An aliquot of 75 µl was added to 75 µl of a serial dilution of the peptide in water in a 96-well microtiter plate (cone type) and incubated for 1 h at 37°C with constant shaking. After removing of the unlysed erythrocytes by centrifugation (1000 rcf, 5 min) 100 µl of the supernatant was transferred to a new microtiter plate and hemoglobin absorption was determined at 450 nm (background correction at 750 nm). 100% lysis was determined by adding 1 µl of a 10% Triton X-100 solution prior to centrifugation. The hemolytic units were calculated as reciprocal value of the peptide concentration, where 50% leakage was observed (i.e. the volume in µl of erythrocyte solution which is lysed to 50% per µg of peptide). Values below 3 hemolytic units are extrapolated. The values are given in Fig. 51. As can be seen, P50 dim and EALA-P50 exhibited the highest pH specific acitivity with regard to lysis of cells and/or release of larger molecules such as hemoglobin. The p50 monomers P50mal and P50 SS-Py had lower activity. Melittin showed the highest activity, which was, however, not specific for acidic pH.

d) Preparation of DNA combination complexes:

DNA complexes were prepared by first mixing 6 µg of pCMVL-DNA in 150 µl HBS with 4 µg TfpL290 in 150 µl HBS and subsequent mixing with 4 to 20 µg of poly(L)lysine290 in 100 µl HBS after 30 min at room temperature. After further 30 min incubation at room temperature, 0.3 to 30 µg of peptide in 100 µl HBS was added and incubated for another 30 min. The optimal amount of endosomolyic agent was determined in preliminary titrations by assaying the resulting gene transfer efficiency (see Table 3 for gene transfer to BNL CL.2 cells). Simultaneous addition of pLys and endosomolytic agent as well as the use of larger volumes for the complex preparation (1.5 ml final volume) was shown to give comparable (or better) transfection efficiencies. In these experiments, the non-peptidic amphipathic substances desoxycholic acid and oleic acid were also shown to augment DNA delivery.

e) Transfection of cells:

Adherent cell lines (BNL CL.2 hepatocytes or NIH 3T3 cells, respectively) were grown in 6 cm dishes for 1 to 2 days prior to transfection (DMEM medium with 10% FCS; 300,000 cells per dish). The medium was removed and 1.5 ml of DMEM + 2% FCS and 500 µl of the DNA complexes were added. Alternatively, 0.5 ml DMEM + 6% FCS and 1.5 ml of DNA complexes was used. After 4 h incubation 2 ml DMEM (18% FCS) was added. (It was found that, alternatively, the transfection medium can be replaced by 4 ml of DMEM with 10% FCS). Harvesting of cells and luciferase assays were performed 24 h after transfection as described previously. The light unit values shown, represent the total luciferase activity of the transfected cells. Transfection of BNL CL.2 hepatocytes is shown in Fig. 52:

Fig. 52A: DNA complexes were prepared by first mixing 6 µg of pCMVL-DNA in 250 µl HBS with 4 µg TfpL290 in 250 µl HBS and subsequent mixing with 20 µg of

poly(L)lysine290 in 750 µL HBS after 30 min at room temperature. After further 30 min incubation at room temperature, indicated amounts of peptides in 250 µl HBS were added. After an incubation for another 30 min, complexes were mixed with 0.5 ml DMEM plus 6% FCS and added to 450,000 cells.

Fig. 52B: DNA complexes were prepared as follows. A solution of 6 µg of pCMVL-DNA in 500 µl HBS was mixed with 4 µg TfpL290 in 250 µl HBS and left for 30 min at room temperature. A 500-µl solution of 20 µg of poly(L)lysine290 in HBS was mixed with indicated amounts of peptides in 250 µl HBS and immediately added to the TfpL/DNA mixture. After further 30 min incubation at room temperature the complexes were mixed with 0.5 ml DMEM plus 6% FCS and added to 450,000 cells. Harvesting of cells 24 h after transfection and luciferase assays were performed as described previously.

The experiments carried out with NIH3T3 cells are shown in Fig. 53. The preparation of complexes according to A) and B) was the same as for the transfection of BNL CL.2 cells.

In the cell culture experiments the peptides P50 dim and EALA-P50 exhibited the highest activity, EALA and GLF had medium activity, whereas P50 monomers and melittin had low activity.

Example 38

Gene transfer using a synthetic non-viral peptide with an oligolysine C-terminal extension

A peptide with the sequence (SEQ ID NO:4) Met Ala Gln Asp Ile Ile Ser Thr Ile Gly Asp Leu Val Lys Trp Ile Ile Asp Thr Val Asn Lys Phe Thr Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys was synthesized and purified according to the method described in the previous Example. This peptide is derived from the δ-toxin of

Staphylococcus aureus (SEQ ID NO:3) Met Ala Gln Asp Ile Ile Ser Thr Ile Gly Asp Leu Val Lys Trp Ile Ile Asp Thr Val Asn Lys Phe Thr Lys Lys, which is known to possess specificity for membrane disruption at acidic pH by extending this peptide by an additional 10 lysine residues.

DNA complexes were prepared by first mixing 6 µg of pCMVL-DNA in 170 µl HBS with 4 µg TfpL290 in 170 µl HBS and subsequent mixing with approximately 3 µg of peptide in 170 µl HBS after 30 min at room temperature. After an incubation of another 30 min at room temperature, complexes were mixed with 1.5 ml DMEM plus 2 % FCS and added to 450,000 BNL CL.2 hepatocytes. After 2 h, 2 ml of DMEM plus 20 % FCS were added. Harvesting of cells 24 h after transfection and measuring luciferase activity were performed as described in the previous Examples. The luciferase activity corresponding to the total extract was 481,000 light units.

Example 39

Transfection of hepatocytes in the presence of melittin-peptides with a C-terminal oligo-Lysine-tail

Peptides of the sequences (N to C terminus) (SEQ ID NO:12) Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro Ala Leu Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys (designated mel 1)
and (SEQ ID NO:13) Gly Ile Gly Ala Val Leu Glu Val Leu Glu Thr Gly Leu Pro Ala Leu Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys (acidic mutant, designated mel 2) were synthesized as described in Example 38.

DNA complexes were prepared by first mixing 6 µg of pCMVL-DNA in 170 µl HBS with 4 µg TfpL290 in 170 µl HBS and subsequent mixing with approximately 3 µg of peptide

mel1 or 5 µg of mel2 in 170 µl HBS after 30 min at room temperature. After an incubation for another 30 min, complexes were mixed with 1.5 ml DMEM plus 2 % FCS and added to 450,000 BNL CL.2 cells, cultivated as described in Example 38. Harvesting of cells 24 h after transfection and luciferase assay were performed as described. The luciferase activity corresponding to the total extract were 9200 light units (in the case of mel1) and 9400 light units (in the case of mel2).

Example 40

Interferon alpha expression in HeLa cells

HeLa cells (5×10^5 cells per 6 cm dish) were transfected with plasmid pAD-CMV1-IFN encoding human interferon alpha2c under the control of the CMV enhancer/promoter sequence (the plasmid is described in DE 40 21 917 A. pADCMV1-IFN was obtained by recloning the HindIII-XbaI IFN-α2c insert into pAD-CMV1). Samples of 6 µg DNA in 330 µl HBS were mixed with 8 µg of TfpL in 330 µl HBS and allowed to stand for 30 minutes at room temperature. Samples 6-10 contained only 4 µg of TfpL and after the first 30 minutes incubation an aliquot of P16pL (20 µg) in 160 µl of HBS was added to both samples 6 and 7 and an aliquot of pLys 290 (20 µg) was added to samples 8, 9 and 10. After an additional 30 minute incubation, aliquots of 160 µl of HBS containing 10 µl (sample 8) or 50 µl (sample 9 and 10) of free P16 were added (for synthesis of P16 and P16pL, see Example 13). After an additional 30 minute incubation, the samples were supplied to HeLa cells in 2 ml of DMEM/2%FCS in the presence of the following additional compounds. Sample 2, 7 and 10 contained 100 µM chloroquine, samples 3 and 4 contained 5 and 15 µl of adenovirus dl312, (1×10^{12} particles/ml), sample 5 contained 15 µl of the same virus, psoralen-inactivated.

(As controls for adenovirus stimulation of endogenous interferon production, samples 11,12 and 13 were treated with aliquots of virus equal to samples 3, 4 and 5). At 2 hours after transfection 5 ml of fresh medium DMEM+10%FCS were added to the cells. At 48 hours after transfection the medium was removed and replaced with 2 ml of fresh DMEM+10% FCS. This medium was harvested at 72 hours post transfection and an ELISA analysis for interferon alpha was performed as described in DE 40 21 917. The interferon alpha levels (in ng/ml) are displayed in Fig. 54.

TfpL functioned poorly to deliver IFN genes to these cells, consistent with the previous observations using luciferase or ß-galactosidase reporter genes. The presence of chloroquine generated a detectable signal (ca. 7 ng/ml, sample 2), but adenovirus dl312 stimulated DNA delivery in a dose dependent fashion (samples 3 and 4). Treating these cells with comparable quantities of virus in the absence of IFN DNA complexes did not result in a detectable interferon signal (samples 11 and 12). Transfection with the synthetic influenza-derived endosomolytic peptide P16(see Example 13) as a conjugate (sample 6,7) or as a peptide ionically bound to the surface of TfpL/DNA complexes (samples 8, 9 and 10, for binding of peptides see Example 37) generated detectable levels of interferon production, which was enhanced with the peptide conjugate in the presence of chloroquine (sample 7).

Tab. 1A

Transfection methods

CELL TYPE	TfpL (A.)	TfpL +Chloroquine (A.)	TfpL +free AdenoV (B.)	TfpL +bound AdenoV (E.)	TfpL +Influ-pLys (F.)
Complex:					
Hepatocytes					
HepG2	-	+/-	+++	++++	+++
BNL.CL2	-	+/-	++++	++++	
primary	-	-	-	++	
Myoblasts/Myotubes					
C2C12	+/-		+/-	++++	
G8	+/-		+/-	++++	
primary				+++	
Fibroblasts					
3T3	+	++	+++	++++	+++
Mov-13	+/-	+/-	+	++++	+++
mouse L-cells	+/-	+	+	++++	+++
primary					
endothelial cells					
pig aorta		+/-	+	++	+/-
human neuroblastoma cell line					
GI-ME-N	+/-	+/-	+++	++++	+
HeLa cells	+	+	+++++	++++	+++

Tab. 1B

Mouse ES cells.					
CCE	+/-		+	++	
Bruce 4	+/-		++	+++	
erythroid cells					
K562	-	++++	+	+++++	+
chicken HD3	++	++++	+++	+++++	
bone marrow					
mouse	-		-	++	
chicken	+/-		+	+++	
EBV-transformed Human B-cells	-	-	-	+++	+
mouse plasma cell lines (MPC11.SP2/0)		+	++	+++	

+/- luciferase activity 1000-10.000 light units per 10^6 cells
\+ 10.000-10^6 light units
++ 1-5x10^6 light units
+++ 5-50x10^6 light units
++++ 50-10x10^6 light units
+++++ >100x10^6 light units

TfpL Transferrin-Polylysine
AdenoV Replication-defective Adenovirus dl312
Influ-pLys Influenza HA-2 N-terminal peptide-polylysine

Tab. 2A

Membrane-active proteins

Viral fusion proteins				
N-terminal fusion sequence				
coated viruses			White,1990; Takahashi,1990	
Influenza virus	Myxoviridae	HA2	pH 5	Hoekstra, 1990
VSV	Rhabdoviridae	G	pH 5	Gong et al., 1990
Vaccinia virus		14 kDa	pH 5	Hoekstra 1990;
Sendai virus	Parainfluenzav.	F1	pH 7	Gething et al., 1978
measles virus	Parainfluenzav.	F	pH 7	Takahashi, 1990
HIV	Retroviridae	gp41	pH 7	Slepushkin et al., 1992
SIV	Retroviridae	gp41	pH 7	Ruysschaert u.Vandenbranden, 1991; Franchini, 1989
uncoated viruses				
polio virus	Enteroviridae	vp1	pH 5	Fricks and Hogle, 1990
Coxsackie virus	Enteroviridae	vp1	pH 5	Gething et al., 1978
Rhinovirus		vp1	pH 5	
Internal fusion sequence				
coated viruses				
Semliki Forest V.	Togaviridae	E1	pH 5	White, 1990
Sindbisvirus				White, 1990
uncoated viruses				
Rhesus Rotavirus		vp5		Mackow et al., 1988

Toxins of microorganisms				
Streptolysin O	Streptococcus sulfhydryl-activated binds to cholesterol 20-80mer, 15 nm Pores	69 kDa	pH 7	Kehoe et al., 1987
Listeriolysin O	L. monocytogenes sulfhydryl-activated, binds to cholesterol related to C9 and Streptolysin O	60 kDa	pH 5	Geoffroy et al., 1987
α-Toxin	Staphylococcus ampipathic surface ß-sheet structure hexameric lesions 2 nm pores	34 kDa	pH 7	
haemolysin	E. coli 8 amphip. Helixes	416 AA	pH 7	Oropeza-Wekerle et al., 1992
haemolysin	Trypanosoma cruzi analogy to perforins related to C9	75 kDa	pH 5	Andrews et al., 1990
Vertebrate immune system				
Perforin	cytotoxic T-cells Ca^{2+}-dependent membrane insertion			Ojcius and Jung, 1991
Complement	C9 (MAC C5b-8,91-4) hollow protein cylinder 10 nm channel, highly-regulated activity			Bhakdi and Tranum-Jonsen, 1991; Esser, 1991
Sperm-egg fusion protein				
PH-30	α-sub-unit of the surface protein, internal fusion sequence		pH 7	Blobel et al., 1992

Tab. 2B

Tab. 2C

Membrane-active peptides

Defence toxins			
Melittin	26AA	amph. α-Helix	Blondelle u. Houghten, 1991; Dempsey et al., 1991; Ikura und Inagaki, 1991; Argiolas u. Pisano, 1985
bee venom		pro-rotated	
Bombolitin	17AA	amph. α-Helix	Argiolas u. Pisano, 1985
bumblebee venom			
Mastoparan	14AA	amph. α-Helix	Argiolas u. Pisano, 1985
wasp venom			
Crabrolin	13AA	amph. α-Helix	Argiolas u. Pisano, 1985
hornet venom			
Pardaxin	33AA	amph. α-Helix	Shai et al., 1990
moses sole fish (shark repellent)		pro-rotated	
Cecropins	37AA	amph. α-Helix	Essor, 1991
Insects		pro-rotated	
(Humoral immune system, silk moth)			
Maganin	23AA	amph. α-Helix	Marion et al., 1988
skin of Xenopus laevis			
Alamethicin	15-24AA	amph. α-Helix	Essor, 1991
Fungus (Trichoderma viride)		α-amino-butyric acid	
Bacterial toxins			
δ-Toxin	26AA	amph. α-Helix	Thiaudiere et al., 1991;
Staphylococcus aureus		acid-induced	Alouf et al., 1989
Amoebapor	25AA	amph. α-Helix	Laippe et al., 1991
Entamoeba histolytica		acid-induced	
Vertebrate Immune system			
Defensins	29-34AA	β-sheet (SS-bridge)	Lehrer et al., 1991
polynuclear neutrophils			

Tab. 3

Transfection of 8NL CL.2 cells
(6 µg pCMV-L DNA, 4 µg TfpL290)

pLys		0 µg	0.3 µg	1 µg	3 µg	10 µg	20 µg	30 µg
0 µg	P50 dm	160	330	540	300			
	GLF		490	290	340			
	Melittin		0	0	0	70	-	425
4 µg	P50 dm	3 100		200	430	180	410	
	GLF		670	600	170			
	EALA		3 140	150	560			
10 µg	P50 dm	5 700				760	1 330	3 424 800
	GLF		1 950	16 600	217 000	215 000	1 980	
	EALA		2 120	16 800	179 300	181 700	76 360	
20 µg	P50 dm	3 200			23 300	185 100	7 054 600	9 344 000
	GLF				418 400	320 600	294 200	
	EALA				191 000	181 000	273 600	
	Melittin				6 545			
	Desoxy-cholic acid			6 730	34 700	16 000		
	Oleic acid			12 200	11 900	4 100		

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SEQUENCE LISTING

(ii) TITLE OF INVENTION: Composition for introducing nucleic acid complexes into higher eucaryotic cells

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Leu Phe Glu Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp
1 5 10
Glu Gly Met Ile Asp Gly Gly Gly Cys
15 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 Amino acids

(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp
1               5                   10
Glu Gly Met Ile Asp Gly Gly Gly Cys
15                  20
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Ala Gln Asp Ile Ile Ser Thr Ile Gly Asp Leu Val Lys
1               5                   10
Trp Ile Ile Asp Thr Val Asn Lys Phe Thr Lys Lys
15                  20                  25
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 Amino acids

(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ala Gln Asp Ile Ile Ser Thr Ile Gly Asp Leu Val Lys
1               5                  10
Trp Ile Ile Asp Thr Val Asn Lys Phe Thr Lys Lys Lys Lys
15                  20                  25
Lys Lys Lys Lys Lys Lys Lys Lys
    30                  35
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Trp Glu Ala Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala
1               5                  10
Glu His Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu
15                  20                  25
Ala Ala Gly Gly Ser Cys
 30
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Leu Phe Gly Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu
1 5 10
Ala Glu His Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala
15 20 25
Leu Ala Ala Gly Gly Ser Cys
30 35

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Gly Leu Phe Gly Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu
1               5                  10
Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala
15                  20                  25
   Leu Ala Ala Gly Gly Ser Cys
   30                  35
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Gly Leu Phe Glu Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala
1               5                  10
Glu Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu
15                  20                  25
Ala Ala Gly Gly Ser Cys
   30
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single

(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp
1               5                  10
Glu Gly Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu
15                  20                  25
Ala Ala Gly Gly Ser Cys
    30
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Gly Leu Phe Glu Ala Ile Glu Gly Phe Ile Glu Asn Gly Trp
1               5                  10
Glu Gly Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu
15                  20                  25
Ala Ala Gly Gly Ser Cys
    30
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Leu Phe Glu Ala Ile Glu Gly Phe Ile Glu Asn Gly Trp
1 5 10
Glu Gly Met Ile Asp Gly Gly Gly Cys
15 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 Amino acids

(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro
1 5 10
Ala Leu Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln Lys Lys
15 20 25

Lys Lys Lys Lys Lys Lys Lys Lys
30 35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 Amino acids
(B) TYPE: Amino acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Ile Gly Ala Val Leu Glu Val Leu Glu Thr Gly Leu Pro
1 5 10
Ala Leu Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln Lys Lys
15 20 25
Lys Lys Lys Lys Lys Lys Lys Lys
30 35

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A composition for the transfection of higher eucaryotic cells with a complex of nucleic acid and a substance having affinity for nucleic acid, which substance is optionally coupled with an internalizing factor for said cells, characterized in that said composition contains an endosomolytic agent which has the ability of being internalized into the cells which are to be transfected, either per se or as a component of the nucleic acid complex, and of releasing the contents of the endosome, in which the complex is located after entering the cell, into the cytoplasm.

2. A composition according to claim 1, characterized in that the endosomolytic agent is a virus or a virus component which is an internalizing factor per se for said cells.

3. A composition according to claim 1, characterized in that the endosomolytic agent has a nucleic acid binding domain or is bound to a substance having affinity for nucleic acid and has the ability of being internalized into said cells as a component of the conjugate/nucleic acid complex, said complex further optionally comprising an internalizing factor for the cells.

4. A composition according to claim 3, characterized in that the endosomolytic agent is covalently bound to a substance having affinity for nucleic acid.

5. A composition according to claim 3, characterized in that the endosomolytic agent is non-covalently bound to the substance having affinity for nucleic acid.

6. A composition according to claim 5, characterized in that the binding is effected via a biotin-streptavidin bridge.

7. A composition according to claim 5, characterized in that the binding is effected ionically.

8. A composition according to claim 3, characterized

in that the endosomolytic agent is bound to the substance having an affinity for nucleic acid direct via a nucleic acid binding domain.

9. A composition according to claim 3, characterized in that the endosomolytic agent is a virus or a virus component.

10. A composition according to claim 2 or 9, characterized in that the endosomolytic agent is an adenovirus.

11. A composition according to claim 10, characterized in that the adenovirus is a mutant.

12. A composition according to claim 11, characterized in that the adenovirus is a replication-incompetent mutant.

13. A composition according to claim 12, characterized in that the adenovirus has one or more mutations and/or deletions in the E1A region.

14. A composition according to claim 10, characterized in that the adenovirus is inactivated.

15. A composition according to claim 14, characterized in that the adenovirus is inactivated by short wave UV, UV/psoralen or formaldehyde.

16. A composition according to claim 9, characterized in that the virus component is one or more adenovirus proteins.

17. A composition according to claim 2 or 9, characterized in that the virus is a picorna virus.

18. A composition according to claim 17, characterized in that the picorna virus is a rhinovirus.

19. A composition according to claim 18, characterized in that the rhinovirus is inactivated.

20. A composition according to claim 3, characterized in that the endosomolytic agent is not an internalizing factor per se for the cell and that the nucleic acid complex further comprises an internalizing factor for said cell which factor is bound to a substance having affinity for nucleic acid.

21. A composition according to claim 20, characterized in that the endosomolytic agent is a virus or a virus component which is not an internalizing factor for a human cell.

22. A composition according to claim 21, characterized in that the endosomolytic agent is a virus which is infectious for a species other than human.

23. A composition according to claim 22, characterized in that the virus is an adenovirus.

24. A composition according to claim 23, characterized in that the adenovirus is avian.

25. A composition according to claim 24, characterized in that the adenovirus is the Chick Embryo Lethal Orphan virus.

26. A composition according to claim 20, characterized in that the endosomolytic agent is an optionally modified endosomolytic viral peptide.

27. A composition according to claim 26, characterized in that the endosomolytic peptide is an influenza-hemagglutinin HA2 peptide.

28. A composition according to claim 27, characterized in that the peptide has the sequence Gly-Leu-Phe-Glu-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-Trp-Glu-Gly-Met-Ile-Asp-Gly-Gly-Gly-Cys.

29. A composition according to claim 27, characterized in that the peptide has the sequence Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-Trp-Glu-Gly-Met-Ile-Asp-Gly-Gly-Gly-Cys.

30. A composition according to claim 20, characterized in that the endosomolytic agent is a non-viral, optionally modified natural or a synthetic peptide.

31. A composition according to claim 30, characterized in that the peptide has the sequence Gly Leu Phe Glu Ala Ile Glu Gly Phe Ile Glu Asn Gly Trp Glu Gly Met Ile Asp Gly Gly Gly Cys.

32. Composition according to claim 30, characterized in that the peptide is a homo- or heterodimer of the

peptide defined in claim 31.

33. Composition according to claim 32, characterized in that the peptide is a homodimer.

34. Composition according to claim 32, characterized in that the peptide is a heterodimer which contains the sequence Gly Leu Phe Glu Ala Ile Glu Gly Phe Ile Glu Asn Gly Trp Glu Gly Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys.

35. Composition according to claim 30, characterized in that the peptide has the sequence Trp Glu Ala Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu His Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys.

36. A composition according to claim 30, characterized in that the peptide has the sequence Gly Leu Phe Gly Ala Leu Ala Glu Ala Leu Ala Glu Ala Leu Ala Glu His Leu Ala Glu Ala Leu Ala Glu Ala Leu Glu Ala Leu Ala Ala Gly Gly Ser Cys.

37. A composition according to claim 26 or 30, characterized in that the peptide has a nucleic acid binding domain.

38. Composition according to claim 37, characterized in that the peptide has an oligolysine extension.

39. A composition according to claim 3 or 20, characterized in that the internalizing factor is transferrin.

40. A composition according to claim 3 or 20, characterized in that the internalising factor is a ligand for T-cells.

41. A composition according to claim 3 or 20 characterized in that the internalising factor is a ligand for B-cells.

42. A composition according to claim 41, characterized in that the internalising factor is an immunoglobulin.

43. A composition according to claim 41, characterized in that the ligand is an anti-immunoglobulin.

44. A composition according to claim 3 or 20,

characterized in that the internalizing factor is a ligand for hepatocytes.

45. A composition according to claim 44, characterized in that the internalizing factor is a ligand for the asialoglycoprotein receptor.

46. A composition according to claim 45, characterized in that the internalizing factor is a tetra-galactose-polylysine.

47. A composition according to claim 3 or 20, characterized in that the internalising factor is a lectin.

48. A composition according to claim 3 or 20, characterized in that the internalising factor is a low-density lipoprotein.

49. A complex as a constituent of a composition of any of the claims 3 to 48, characterised in that it comprises one or more nucleic acids to be expressed in the cell, an endosomolytic agent which originally has a nucleic acid binding domain or which is bound to a substance having affinity for nucleic acid, which complex optionally further comprises an internalizing factor which is bound to a substance having affinity for nucleic acid.

50. A complex according to claim 49, characterized in that it contains a therapeutically active nucleic acid.

51. A complex according to claim 50, characterized in that said nucleic acid comprises one or more DNA molecules which are active in gene therapy.

52. A complex according to claim 51, characterized in that said DNA molecules encode cytokines.

53. A complex according to claim 50, characterized in that said nucleic acid comprises a nucleotide sequence from which RNA molecules which specifically inhibit cell functions can be transcribed.

54. A complex according to claim 49, characterized in that the substance having affinity for nucleic acid is an organic polycation.

55. A complex according to claim 54, characterized in that the polycation is polylysine.

56. A complex according to claim 49, characterized in that the endosomolytic agent and the internalizing factor are both bound to the same substance having affinity for nucleic acid.

57. A complex according to claim 56, characterized in that the endosomolytic agent and the internalizing factor are bound to polylysine.

58. A complex according to claim 57, characterized in that it further comprises non-covalently bound polylysine.

59. A conjugate as a constituent of a complex of any of the claims 49 to 58, characterized in that said conjugate comprises an endosomolytic agent and a substance having affinity to nucleic acid.

60. A conjugate according to claim 59, characterized in that the endosomolytic agent is as defined in any one of claims 4 to 7 or 9 to 38.

61. Endosomolytic peptide suitable as a constituent of the composition according to claim 37, characterized in that it is an artificial peptide which has an endosomolytic domain and a nucleic acid binding domain.

62. Peptide according to claim 61, characterized in that the nucleic acid binding domain is an oligolysine extension.

63. A process of preparing a conjugate of claim 60, characterized in that a virus or a (poly)peptidic endosomolytic agent and a polyamine are enzymatically coupled in the presence of a transglutaminase.

64. A process of preparing a conjugate of claim 60, characterized in that a virus or a (poly)peptidic endosomolytic agent and a polyamine are chemically coupled.

65. A process of preparing a conjugate of claim 61, characterised in that a virus or a virus component is modified with biotin, and bound to a streptavidin-

coupled polyamine.

66. A process for introducing nucleic acid into higher eucaryotic cells, characterized in that the cells are treated with a composition according to any of the claims 1 to 48.

67. A process according to claim 66, characterized in that the cells are human cells.

68. A process according to claim 67, characterized in that the cells are tumor cells.

69. A process according to claim 67, characterized in that the cells are myoblasts.

70. A process according to claim 67, characterized in that the cells are fibroblasts.

71. A process according to claim 67, characterized in that the cells are hepatocytes.

72. A process according to claim 67, characterized in that the cells are endothelial cells.

73. A process according to claim 72, characterized in that the cells are respiratory tract cells.

74. A process according to claim 67, characterized in that the cells are bone marrow cells.

75. A process according to one of claims 66 to 74, characterized in that the nucleic acid of the composition is active in gene therapy.

76. A process according to claim 75, characterized in that the nucleic acid encodes Factor VIII.

77. A process according to claims 68 and 75, characterized in that the tumor cells are treated with the composition ex vivo and that said DNA encodes one or more immune modulating substances, preferably cytokines.

78. A process for producing a heterologous protein in a higher eucaryotic cell, characterized in that the cells are treated with a composition of claim 1, the nucleic acid comprising a DNA sequence encoding the desired protein, the cells are cultivated under conditions suitable for expression of the protein, and the protein is recovered.

79. A pharmaceutical preparation comprising a composition of any of the claims 1 to 48, containing a therapeutically active nucleic acid and a pharmaceutically acceptable carrier.

80. Transfection kit containing a carrier unit in which there are two or more containers, a first container containing a substance having an affinity for nucleic acid which is optionally coupled to an internalising factor for a higher eucaryotic cell and a second container contains an endosomolytic agent which is capable of penetrating per se into higher eucaryotic cells and releasing the contents of endosomes into the cytoplasm.

81. A transfection kit containing a carrier unit in which there are two or more containers, a first container containing a substance having an affinity for nucleic acid which is optionally coupled to an internalising factor for a higher eucaryotic cell and a second container contains a substance having an affinity for nucleic acid which is coupled to an endosomolytic agent capable of penetrating into higher eucaryotic cells as a component of a nucleic acid complex and releasing the contents of endosomes into the cytoplasm.

82. Transfection kit according to claim 81, characterized in that it contains in a first container transglutaminase-coupled adenovirus-polylysine conjugate.

83. Transfection kit containing a carrier unit in which there are two or more containers, a first container containing a biotin-modified endosomolytic agent and a second container containing a streptavidin-modified substance having an affinity for nucleic acid.

84. Transfection kit according to claim 83, characterized in that the first container contains biotinylated adenovirus and a second container contains streptavidin-polylysine.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 92/02234

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[5] C12N15/87; A61K48/00; C12N7/04; A61K47/48
C07K13/00; C12N15/34; C12N15/41; C12N15/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl.[5] C12N; C07K; A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Vol. 88, No. 19, 1 October 1991, WASHINGTON US pages 8850 - 8854 CURIEL, D.T. ET AL: 'Adenovirus enhancement of transferrin-polylysine-mediated gene delivery' see the whole document --- -/--	1,2, 9-13,18, 21,40, 50-52, 55-60, 62,65-69 74,77, 78,99, 101-103, 109-117

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 January 1993 (06.01.93)	21 January 1993 (21.01.93)
Name and mailing address of the ISA/ European Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 92/02234

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOLECULAR AND CELLULAR BIOLOGY Vol. 4, No.8, August 1984, pages 1528 - 1533 SETH, P. ET AL. 'Evidence that the penton base of adenovirus is involved in potentiation of toxicity of Pseudomonas exotoxin conjugated to Epidermal Growth Factor' see the whole document	1-18,21, 22,27, 38-74, 77-83, 96-117
Y	JOURNAL OF VIROLOGY Vol.51, No. 3, September 1984, pages 650 -655 SETH, P. ET AL. 'Role of a low-pH environment in adenovirus enhancement of the toxicity of a Pseudomonas exotoxin-Epidermal Growth Factor conjugate' see the whole document	1-18,21, 22,27, 38-74, 77, 96-117
Y	GENE Vol. 63, No. 2, 1988, AMSTERDAM NL pages 321 - 330 TIKCHONENKO, T.I. ET AL. 'Transfer of condensed viral DNA into eukaryotic cells using proteoliposomes' see the whole document	1,2,9, 27,28, 83,84
Y	GENE Vol. 84, No. 2, 14 December 1989, AMSTERDAM NL pages 429 - 438 GOULD-FOGERITE, S. ET AL. 'Chimerasome-mediated gene transfer in vitro and in vivo' see the whole document	1,2,9, 27,28, 83,84
Y	JOURNAL OF GENERAL VIROLOGY Vol. 69, No.8, August 1988, UK pages 1847 - 1857 WHARTON, S.A ET AL. 'Membrane fusion by peptide analogues of Influenza Virus haemagglutinin' see the whole document	1,2,9, 27,28, 83,84
Y	EP, A, 0 388 758 (BOEHRINGER INGELHEIM INTERNATIONAL G.M.B.H.) 26 September 1990 see the whole document	1,2,9, 27,28, 83,84

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 92/02234

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Vol. 89, No. 13, 1 July 1992, WASHINGTON US pages 6094 - 6098 COTTEN, M. ET AL. 'High-efficiency receptor-mediated delivery of small and large (48 kilobase gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles' see the whole document	1-2, 9-16,18, 21,40, 50-52, 55-60, 62,65-72 77,78, 99, 101-103, 109-117
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Vol. 89, No. 13, 1 July 1992, WASHINGTON US pages 6099 - 6103 WAGNER, E. ET AL. 'Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes' see the whole document	1-2, 9-16,18, 21,40, 50-52, 55-60, 62,63 65-72, 74,77,78, 99-102,109, 11-117
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Vol. 89, No. 17, 1 September 1992, WASHINGTON US pages 7934 - 7938 WAGNER, E. ET AL. 'Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle' see the whole document	1-2,21, 27-32, 40 50-52, 55-61, 65-77, 78,83 84-88,99, 101-103,106, 109-117
P,Y	WO, A, 9 117 773 (BOEHRINGER INGELHEIM INTERNATIONAL G.M.B.H.) 28 November 1991 see the whole document	1,2,9 27,28, 83,84
Y	EP, A, 0 422 543 (GREEN CROSS CORPORATION) 17 April 1991 see the whole document	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9202234 SA 64707

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/01/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0388758	26-09-90	AU-A-	5137290	20-09-90
		CA-A-	2012311	16-09-90
		JP-A-	3200800	02-09-91
WO-A-9117773	28-11-91	DE-A-	4110410	01-10-92
EP-A-0422543	17-04-91	JP-A-	3127622	30-05-91

INTERNATIONALER RECHERCHENBERICHT

PCT/EP 92/02234

Internationales Aktenzeichen

I. KLASSIFIKATION DES ANMELDUNGSGEGENSTANDS (bei mehreren Klassifikationssymbolen sind alle anzugeben)[6]

Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC

Int.Kl. 5 C12N15/87; A61K48/00; C12N7/04; A61K47/48
C07K13/00; C12N15/34; C12N15/41; C12N15/44

II. RECHERCHIERTE SACHGEBIETE

Recherchierter Mindestprüfstoff [7]

Klassifikationssystem	Klassifikationssymbole
Int.Kl. 5	C12N ; C07K ; A61K

Recherchierte nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Sachgebiete fallen [8]

III. EINSCHLAGIGE VERÖFFENTLICHUNGEN [9]

Art.°	Kennzeichnung der Veröffentlichung [11], soweit erforderlich unter Angabe der maßgeblichen Teile [12]	Betr. Anspruch Nr.[13]
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Bd. 88, Nr. 19, 1. Oktober 1991, WASHINGTON US Seiten 8850 - 8854 CURIEL, D.T. ET AL. 'Adenovirus enhancement of transferrin-polylysine-mediated gene delivery' siehe das ganze Dokument	1,2, 9-13,18, 21,40, 50-52, 55-60, 62,65-69 74,77, 78,99, 101-103, 109-117

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° Besondere Kategorien von angegebenen Veröffentlichungen [10]:

"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden

"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

IV. BESCHEINIGUNG

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
06.JANUAR 1993	22.01.93
Internationale Recherchenbehörde	**Unterschrift des bevollmächtigten Bediensteten**
EUROPAISCHES PATENTAMT	CHAMBONNET F.J.

3

III. EINSCHLAGIGE VERÖFFENTLICHUNGEN (Fortsetzung von Blatt 2)

Art °	Kennzeichnung der Veröffentlichung, soweit erforderlich unter Angabe der maßgeblichen Teile	Betr. Anspruch Nr.
Y	MOLECULAR AND CELLULAR BIOLOGY Bd. 4, Nr. 8, August 1984, Seiten 1528 - 1533 SETH, P. ET AL. 'Evidence that the penton base of adenovirus is involved in potentiation of toxicity of Pseudomonas exotoxin conjugated to Epidermal Growth Factor' siehe das ganze Dokument	1-18,21, 22,27, 38-74, 77,83, 96-117
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Y	EP,A,0 388 758 (BOEHRINGER INGELHEIM INTERNATIONAL G.M.B.H.) 26. September 1990 siehe das ganze Dokument	1,2,9, 27,28, 83,84

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III. EINSCHLAGIGE VEROFFENTLICHUNGEN (Fortsetzung von Blatt 2)

Art °	Kennzeichnung der Veröffentlichung, soweit erforderlich unter Angabe der maßgeblichen Teile	Betr. Anspruch Nr.
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III. EINSCHLAGIGE VEROFFENTLICHUNGEN (Fortsetzung von Blatt 2)

Art °	Kennzeichnung der Veröffentlichung, soweit erforderlich unter Angabe der maßgeblichen Teile	Betr. Anspruch Nr.
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ANHANG ZUM INTERNATIONALEN RECHERCHENBERICHT ÜBER DIE INTERNATIONALE PATENTANMELDUNG NR.

EP 9202234
SA 64707

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben.
Die Angaben über die Familienmitglieder entsprechen dem Stand der Datei des Europäischen Patentamts am 06/01/93
Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie		Datum der Veröffentlichung
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Für nähere Einzelheiten zu diesem Anhang : siehe Amtsblatt des Europäischen Patentamts, Nr.12/82